THE EFFECT OF HARD PROTEIN CORONA ON QD NANOPARTICLE TOWARDS SENESCENT CELLS

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UNIVERSITI SAINS MALAYSIA
2019
THE EFFECT OF HARD PROTEIN CORONA ON QD NANOPARTICLE TOWARDS SENESCENT CELLS

by

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Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

August 2019
ACKNOWLEDGEMENT

First and foremost, all praise and thanks is to Allah S.W.T. the Lord of the universe that has given me the strength and opportunity to complete this research. I am heartily thankful to my supervisor Professor, Dr. Azlan Abdul Aziz who was abundantly helpful and offered great assistance, support and guidance. I am extremely grateful to my co-supervisor, Professor, Dr. Morteza Mahmoudi for his valuable guidance through this journey. I would like to express my appreciation to NanoBRI staff, especially Mr. A.S. Navanithan for his guidance.

I owe my deepest gratitude to my beloved parents, for their faith in me, their understanding and endless love and allowing me to be as ambitious as I wanted. It was under their watchful eye that I gained so much drive and an ability to tackle challenges head on.

Lastly, I would like to dedicate this thesis to my beloved mother, Pouran Rezazadeh, who provided me with unfailing support and continuous encouragement throughout my years of study. This thesis would not have been possible without all the sacrifices that my lovely mother has done to enable me to complete my study.

SEYEDEH PARISA FOROOZANDEHASL
School of Physics
Universiti Sains Malaysia
February 2019
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ALF</td>
<td>Artificial lysosomal fluid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>µBCA</td>
<td>Micro bicinchoninic colorimetric assay</td>
</tr>
<tr>
<td>MALDI-TOF/TOF</td>
<td>Matrix assisted laser desorption/ionization–time of flight</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2′-deoxyuridine</td>
</tr>
<tr>
<td>SA-β-Gal</td>
<td>Senescence associated β-galactosidase</td>
</tr>
<tr>
<td>SAHF</td>
<td>Senescence associated heterochromatin foci</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>NRR</td>
<td>Neutral red retention</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>VAC</td>
<td>Volume of acidic compartment</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>HRTEM</td>
<td>High-resolution transmission electron microscopy</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>PDL</td>
<td>Population doubling level</td>
</tr>
<tr>
<td>MDC</td>
<td>Monodansylcadaverine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>NaN₃ + 2DG</td>
<td>Sodium azide + 2-deoxyglucose</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
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<td>3-MA</td>
<td>3-Methyladenine</td>
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KESAN PROTEIN KERAS KORONA PADA NANOPARTIKEL QD TERHADAP SEL PENUAAN

ABSTRAK

Perubatan nano merupakan bidang kajian penting yang mengkaji penggunaan bahan nano dan nanopartikel dalam terapi perubatan dan tujuan diagnostik. Walau bagaimanapun, tidak banyak kajian yang menumpukan pada aspek penuaan yang berkaitan dengan nanomedikal yang mungkin berguna untuk merawat penyakit yang berkaitan dengan penuaan seperti sindrom Werner, sarcopenia dan Alzheimer. Dalam kerja ini, potensi sitotoksik titik kuantum (QD-PEG) dan QD-PEG bersalut protein keras corona (QD-HC) pada sel-sel yang berbeza umur diperiksa. Pada fasa awal kajian, interaksi QD dengan protein dari plasma darah manusia dianalisis. Keputusan telah menunjukkan bahawa corona protein dapat membentuk pada QD-PEG berdasarkan analisis SDS-PAGE, MALDI-TOF/TOF, LC-MS/MS dan μBCA. Pembentukan corona protein keras mengubahsuai sifat fizikokimia QD-PEG berdasarkan analisis TEM, AFM, DLS dan potensi zeta. Telah ditentukan bahawa perubahan kepada sifat-sifat fizikokimia telah menjelaskan keupayaan koloid QD-PEG secara signifikan. Pada tahap berkadar konsentrasi nanopartikel, corona protein keras telah memberi ciri-ciri fotonik dan koloid yang unik kepada QD-PEG yang lebih bersesuaian untuk aplikasi nanomedikal dari segi: (1) dipertingkatkan kestabilan fotonik dalam keadaan pH yang terlampau, (2) rintangan yang lebih besar kepada perubahan dalam medium extracellular yang mendorong penumpuan dan pemendapan graviti, dan (3) meningkatkan keteguhan QD-PEG daripada kemusnahan dan larut lepas bahan terasnya dalam keadaan pH yang terlampau. Dalam fasa kedua kajian, kaedah penuaan yang berbeza telah digunakan untuk
membangunkan model senescent sel fibroblast (IMR90) dan sel epitelium (CCD841CoN). Hasilnya telah menunjukkan bahawa model senescent untuk sel IMR90 dan sel CCD841CoN telah berjaya dibangunkan. Dalam fasa akhir kajian ini, potensi sitotoksik daripada QDs pada sel-sel muda dan uzur dinilai menggunakan ujian WST-1, NRR dan LDH. Keputusan daripada kajian menunjukkan bahawa QDs tidak akut toksik kepada sel muda IMR90 dan sel muda CCD841CoN. Sebaliknya, QDs memberi kesan toksik kepada sel-sel senescent IMR90 dan CCD841CoN dengan tahap yang berbeza pada masa pendedahan yang sama. Data telah menunjukkan bahawa kesan toksik QD-PEG telah menyebabkan kematian nekrotik kepada sel senescent IMR90 dan CCD841CoN melalui permeabilisasi membran lysosom dalam tempoh 24 jam inkubasi. Sel-sel senescent mempunyai tindak balas yang berbeza terhadap kesan-kesan toksik disebabkan oleh QD-HC bergantung kepada kepekatannya. Pada kepekatan QD-PEG yang sama, QD-HC telah menyebabkan kematian sel senescent IMR90 dan CCD841CoN melalui autophagy; manakala pada kepekatan QD-HC yang lebih tinggi, kematian sel secara nekrotik melalui permeabilisasi membran lysosom diperhatikan dalam tempoh 24 jam inkubasi. Penemuan kajian ini akan memberi manfaat kepada para penyelidik dalam bidang perubatan nano untuk merancang eksperimen mereka dengan lebih berkesan selepas menyesuaikan pengaruh protein korona dan perbezaan usia dalam kajian yang memaparkan sistem penyampaian ubat berasaskan nanopartikel yang ditujukan kepada aplikasi terapeutik atau klinikal.
ABSTRACT

Nanomedicine is an important area of study that examines the utilization of nanomaterials and nanoparticles in medical therapy and diagnostic purposes. However, not many studies have focused on the aging related aspect of nanomedical research that could have been valuable in treating aging associated diseases such as Werner syndrome, sarcopenia and Alzheimer’s. In the present work, the cytotoxic potential of PEGylated quantum dots (QD-PEG) and hard protein corona coated QD-PEG (QD-HC) on cells of opposing age groups were examined. In the initial phase of the study, the interaction of QDs with proteins from human blood plasma were analyzed. The results have shown that protein corona was able to form on pristine QD-PEG based on SDS-PAGE, MALDI-TOF/TOF, LC-MS/MS and µBCA analysis. Formation of hard protein corona had transformed its physicochemical properties, which had in turn affected the colloidal stability of QD-PEG in a significant manner. At proportionate levels of nanoparticle concentration, hard protein corona had imbued distinct photonic and colloidal characteristics to QD-PEG that were better suited for nanomedical applications in terms of: (1) enhanced photostability at extreme pH conditions, (2) greater resistance to changes in extracellular medium that induces agglomeration and gravitational sedimentation, and (3) increased robustness to degradation and leaching of QDs’ core materials at extreme pH conditions. In the second phase of the study, different aging methods were employed to develop senescent models of fibroblast (IMR90) and epithelial (CCD841CoN) cells. Based on the benchmarks established in the current experiment, senescent models for
IMR90 and CCD841CoN cells were successfully developed. In the final phase of the study, the cytotoxic potential of the QDs on young and senescent cells were assessed and results from the study have demonstrated that the QDs were not acutely toxic on the former. In contrast, the QDs were lethal to senescent cells of both types with varying degree at the same exposure time. The data have shown that QD-PEG were acutely toxic to senescent IMR90 and CCD841CoN cells, leading to lysosomal membrane permeabilization induced necrotic cell death. The senescent cells had divergent response to the toxic effects induced by QD-HC depending on its concentration. At similar concentration of QD-PEG, QD-HC had induced autophagic cell death due to cadmium toxicity and halved the senescent cell population; while, at much higher concentrations of QD-HC, lysosomal membrane permeabilization induced necrosis was observed, resulting in total death of senescent cell population. At all instances, the common denominator was the disruption to the lysosomal activity of senescent cells preceding the loss of its viability. Incidentally, QD disintegration within the lysosomal compartment was determined to be the precursor event leading up to the binary cell deaths. The rate of QD disintegration was the determining factor for the mode of cell death and protein corona was found to effect this process significantly. Deeper introspection has led to the discovery that protein corona had delayed the QDs’ disintegration and consequently had attenuated its cytotoxic potential. The susceptibility of senescent cells to the toxic effects of QDs were attributed to the deterioration of its organelles and disruption in cellular functions relative to the young phenotype. The current findings will benefit researchers in the field of nanomedicine to design their experiments more effectively after adjusting for protein corona influences and age related differences in studies featuring nanoparticle based drug delivery systems geared towards therapeutic or clinical applications.
CHAPTER ONE: INTRODUCTION

1.1 Overview and rationale of the study

The field of nanomedicine is expanding at an astounding rate largely due to the amalgamation of technology from other emerging fields such as nanotechnology, biotechnology and bioconjugation chemistry. The core component of this innovative technology is nanoparticle and its subsequent utility as therapeutic and diagnostic agents in clinical applications makes it an active area of research.

Quantum dots (QDs) are a class of nanoparticle that are widely being used in the biomedical field for diagnostic and imaging applications. Controlled illumination, enhanced resolution and greater resistance to photobleaching relative to the conventional stains are some of the qualities that made QDs an invaluable tool in live imaging of small animals and humans. The delivery of these QDs were mostly administered intravenously at the target site and tracked throughout the circulatory system in the body. As a consequence, the QDs will encounter blood plasma proteins that adsorb onto the circulating nanoparticles either reversibly or irreversibly depending on the affinity of the proteins to the nanoparticle. This phenomenon of protein adsorption is known as ‘The Vroman Effect’ and the adsorbing proteins are denoted as protein corona (Lesniak et al., 2012; Mahmoudi, 2018). Ultimate manifestation of the interaction between these two entities is the modification of QD’s physicochemical properties. This is an undesirable outcome as the QDs are essentially tailored to perform specific functions such as targeted entry into the cells and even targeted sites within the cells or organelles. As such, methods
to circumvent this effect or incorporate it as part of the delivery strategy will greatly benefit in increasing the targeting yield and efficacy of the QDs while inadvertently improving its imaging resolution. In the first phase of this study, the interaction of QDs with proteins from human blood plasma were analysed.

Another serious consideration when administering QDs to living organisms are its toxic effects on the cell, which creates a potential liability for its use. QDs can be toxic to cells due to intrinsic factors such as its core chemical composition, surface chemistry, size distribution and colloidal stability. To study the toxic effects and optimize the parameters for safe administration of QDs, in vitro cellular models can be utilized. These in vitro models are useful tools to quantitatively study the toxic effect of nanoparticles on different types of cultured cells. Apart from the potential of QDs to induce deleterious effects on the cells, the cellular model selected for cytotoxicity evaluation may have an impact on the final outcome of the study.

Some of the cellular models that were typically employed for cytotoxicity testing of nanoparticles are fibroblast, epithelial, endothelial, macrophage, cancer and stem cells. The prerequisite for selecting the in vitro cellular models is to ideally represent the in vivo conditions. However, the age of the cell is an overlooked factor in many studies and the interaction of nanoparticles with ageing cells were rarely addressed, if any in the cytotoxicity studies. By using senescent cell models along with the non-senescent cells of similar genotype, the conclusion of the study will epitomize heterogeneity and reduced
biasness. Therefore, age related studies will provide a more comprehensive understanding of nanoparticle toxicity as it reflects the diversity in the general population. In the second phase of the study, senescent models of fibroblast and epithelial cells were developed using established cell ageing methods to assess the cytotoxic potential of QDs.

Principal goal of the current study was to gauge the effect of protein corona formation on QDs and its subsequent correspondence with cells of different age groups in an *in vitro* setting. Thus, the two tiered experimental approach discussed above were integrated in the final part of the study and the resulting data were meticulously evaluated.

1.2 Research objectives

The current study was undertaken with the following objectives:

1. To characterize and evaluate the impact of hard protein corona on the physicochemical properties of the quantum dots.
2. To establish *in vitro* senescent cell models using fibroblast (IMR90) and epithelial (CCD841CoN) cells.
3. To determine the interaction and cytotoxic potential of pristine and hard corona coated quantum dots on young and senescent cells.
4. To investigate the modality and mechanism of senescent cell death induced by the quantum dots.
1.3 Flow chart of the study

[Flow chart diagram]

Nanoparticles
Qtracker® 705

Characterization of nanoparticles
- Morphology (TEM/HRTEM)
- Particle distribution (DLS)
- Zeta potential (Zetanizer)
- Elemental composition (STEM-EDX)
- Agglomeration/Sedimentation (AFM)

Nanoparticle-hard corona coating preparation
Qtracker® 705 incubated in 55% human blood plasma

Characterization of Nanoparticle-hard corona
- SDS-PAGE
- MALDI TOF/TOF
- LCMS/MS
- BCA assay

Cells
In vivo
- AG04450
- AG02603
- AG02262

-IMR30
- CCD841CoN

Young cells

Cellular aging by replicative senescence

Cellular aging by stress induced premature senescence

Senescent cells

Cellular Biochemical Characterization
- Beta-galactosidase assay
- BrdU assay
- SAHF assay

Interactions

Cytotoxicity assays
- NRU assay
- LDH leakage assay
- WST-1 cell proliferation assay

Cellular uptake study
- Energy dependent cell uptake
- Endocytic cell uptake
- Exocytosis
- Intracellular localization

Statistical analysis of the data
CHAPTER TWO: LITERATURE REVIEW

2.1 Nanoparticles

2.1.1 Characteristics of nanoparticle

Nanoparticles (NPs) are defined as particles with all its three dimensions confined within the range of 1 to 100 nm (Albanese et al., 2012; Docter et al., 2015a; Bhatia, 2016). The growing attention to NP stem from the fact that their mechanical, chemical, optical, electrical, and magnetic properties differs to those of bulk counterparts and these properties can be altered by varying the size (Mahmoudi et al., 2011a; Rahman et al., 2013). Due to their capability of tuning properties for intended requirements, NPs are of significant interest in different fields such as physics, chemistry, engineering, electronics, and biology (Aggarwal et al., 2009; Mahmoudi et al., 2011a; Kharazian et al., 2016; Schöttler et al., 2016). Nanoparticles can be made of inorganic materials like gold, silica, iron oxide, or made of organic polymers including polystyrene (PS), poly (lactic-co-glycolic acid) (PLGA) and polylactic acid (PLA) (Schöttler et al., 2016).

2.1.2 Nanoparticles in biomedical application

The utilization of NPs in biomedical application arise from their inherent properties of small size and high surface to volume ratio (Chinen et al., 2015; Smith et al., 2015). Small size of NP enables them to translocate cross biological barriers and reach subcellular compartment, biological components and those targets that were not possible to access previously such as brain (Saptarshi et al., 2013; Caracciolo et al., 2016). In particular, NPs smaller than 100 nm are able to enter the cells, smaller than 40 nm enter
nucleus of the cells and less than 35 nm can across the blood brain barrier (Dawson et al., 2009). Moreover, high surface to volume ratio of NP make them highly active and more efficiently in interactions with biological component than that of bulk counterpart (Karmali and Simberg, 2011; Westmeier et al., 2015; Polyak and Cordovez, 2016). These interesting properties of nanoparticles make them as a promising multifunctional tool in different medical applications (Docter et al., 2015a).

Nanoparticles are increasingly considered to employ in medical imaging, drug delivery, diagnostic, and hyperthermic therapy purposes (Rahman et al., 2013; Mahmoudi, 2016). Likewise, nanoparticles are highly potential to use as contrast agent in magnetic resonance imaging (MRI), fluorescence spectroscopy and optical imaging (Seeney et al., 2012; Hou et al., 2013; Rizzo et al., 2013; Westmeier et al., 2015). Metal oxides have begun to use in magnetic resonance imaging in 1970s (Rahman et al., 2013). Magnetic nanoparticles have been widely used in magnetic resonance imaging, magnetic particle imaging and magnetic drug targeting as well as in hyperthermia application (Krishnan, 2010; Gräfe et al., 2016). Plasmonic particles such as gold (Au) and silver (Ag) are employed for optical imaging along with laser induced photothermal therapy (de Aberasturi et al., 2015).

Nanoparticles have demonstrated promising features for the delivery of therapeutic drugs to the target site of body (Mirshafiee et al., 2013). In contrast to micron-sized particles that rapidly eliminated by immune system, nanoparticles in drug delivery system can be delivered to all organs (Mause and Weber, 2010; Rak, 2010; Lee et al., 2015). Moreover, NP-based drug delivery shows higher solubility, improved
pharmacokinetics, reduced toxicity, greater biodistribution and increased drug bioavailability which result in fewer side effects and enhancing therapeutic index of drugs (Pautler and Brenner, 2010; Wahajuddin, 2012; Polyak and Cordovez, 2016).

Administration of NP in drug delivery has opened up new opportunities in cancer therapy (van der Meel et al., 2013; Pearson et al., 2014a). Encapsulation of drugs in nanoparticles carrier or nanocapsules have been introduced in cancer therapy as a new promising approach (Albanese et al., 2012; Salvati et al., 2013; Mirshafiee et al., 2016a). Severe side effects that cause by chemotherapeutic drugs due to their high cytotoxicity can be diminished by utilizing nanocarriers. Among chemotherapeutic drugs doxorubicin and paclitaxel were the first drugs which was administrated by nanocarriers (Schöttler et al., 2016).

Numerous nanotherapeutics have already acknowledged clinical approval and several others are currently going through clinical trials (Wolfram et al., 2014). Thus, due to increase application of nanoparticles in nanomedicine, it is crucial to understand their interaction with biological compartment and consequent physiological response to ensure the safe and efficient implementation of nanomedicine (Nel et al., 2009; Mahmoudi et al., 2011b; Walkey and Chan, 2012).
2.2 Nanoparticle protein-corona complex

Due to their large surface to volume ratio, nanoparticles in biological medium tend to lower their high surface energy by interacting with medium components (Monopoli et al., 2011a; Wolfram et al., 2014; Polyak and Cordovez, 2016; Westmeier et al., 2016). Therefore, when NP is dispersed in biological medium physical and chemical interactions arise, leading to formation of new interface between NP and biological component called “bio-nano interface” which is merging of organic and synthetic worlds (Mahmoudi et al., 2011a; Treuel and Nienhaus, 2012; Gunawan et al., 2014; Schöttler et al., 2016). It is now well accepted that upon introduction of NP to biological environment, variety of proteins would cover the surface of NP forming a layer, called “protein corona” (Treuel, 2013; Pearson et al., 2014b; Pozzi et al., 2015; Corbo et al., 2016; Mahmoudi, 2016).

It is noteworthy to mention that it is protein corona that primarily interact with biological component rather than the pristine surface of NP. In particular, protein corona constitute what the biological system actually sees when encounter the NP (Brun and Sicard–Roselli, 2014; Docter et al., 2015b; Liu et al., 2015; Serpooshan et al., 2015; Caracciolo et al., 2016) (Figure 2.1). Protein corona changes the interfacial properties of NP endowing it new identity termed biological identity which is significantly different from its synthetic identity. More specifically, protein corona transforms the synthetic identity of NP to biological identity, making the nanoparticle- protein corona complex to be seen as one entity (Monopoli et al., 2012; Hadjidemetriou et al., 2015; Maiolo et al., 2015; Westmeier et al., 2015; Bigdeli et al., 2016).
This is the biological identity that mediate the interaction with membrane and biological barriers, determining the subsequent physiological responses including cellular uptake, kinetics, transport, biodistribution, signalling, and toxicity of the nanoparticles (Saptarshi et al., 2013; Kelly et al., 2015; Lee et al., 2015; Mahmoudi et al., 2015; Wan et al., 2015; Kharazian et al., 2016). Thus deep understanding of nanoparticle-protein corona complex and its biological implications is a vital step toward safe design of nanoparticle in medical application.

**Figure 2.1:** The nanoparticle–corona complex in a biological environment. (a) It is the nanoparticle–corona complex, rather than the bare nanoparticle, that interacts with biological machinery, here with a cell membrane receptor. (b) Relevant processes (arrows), in both directions (on/off), for a nanoparticle interacting with a receptor. Adapted from (Monopoli et al., 2012).
2.2.1 Composition of the protein-corona

The identities of proteins in corona play a key role in defining the physiological response to NP-protein corona complex. Although, more than 3,700 proteins in the blood plasma compete for binding to the surface of nanoparticle, their abundance in the plasma is not related to their abundance in the protein corona. Furthermore, they are not merely those with the highest affinity for the surface of NP (Ge et al., 2011; Martel et al., 2011; Zhang et al., 2011a; Dufort et al., 2012; Monopoli et al., 2012). It is noteworthy to mention that there is no universal protein corona for all nanoparticle and composition of protein corona is unique to each nanoparticle (Walkey and Chan, 2012).

Walkey and Chan established a trend in composition of protein corona by compiling a list of identified proteins for 63 nanomaterials across numerous studies. They have identified the total of 125 plasma proteins in protein coronas, demonstrating a subset of plasma proteins which adsorb at least to one nanomaterial, termed ‘adsorbome’. A similar trend was observed for all protein coronas in which 2 to 6 proteins for each nanomaterial were adsorbed abundantly, and other adsorbed proteins were low abundance proteins. The proteins that adsorb at high abundance to some nanomaterial are not the same abundant proteins to another. Some proteins may adsorb at high abundance to some nanomaterial but the same proteins adsorb on others with low abundance. They have classified adsorbed blood proteins to two groups; one is included of those proteins that have the capability to adsorb at high abundance to nanomaterial surface but it does not necessarily occur, and another one including of plasma proteins that can only adsorbed at low abundance (Walkey and Chan, 2012).
One protein group which has been extensively identified in protein corona of different nanoparticles is apolipoproteins. This protein which is part of lipoprotein complex, their main role is transporting lipids and cholesterol through the bloodstream (Lynch and Dawson, 2008; Monopoli et al., 2012; Gunawan et al., 2014). Adsorption of apolipoproteins on nanoparticles surface lead to interact with lipoprotein receptors on the cell surface (Wagner et al., 2012; Saptarshi et al., 2013; Tenzer et al., 2013). This characteristic has been exploited to transport the drugs cross the blood–brain barrier (BBB), and reach the central nervous system (CNS) to treat CNS diseases such as Alzheimer's and Parkinson's diseases (Aggarwal et al., 2009; Monopoli et al., 2012; Walkey and Chan, 2012; Gunawan et al., 2014).

Another group of protein that is often recognized in corona profile are complement proteins. This group of proteins are part of innate immune system that helps eliminating the pathogen from the body. The complement system which consist of more than 30 proteins tag the pathogen to be recognized by phagocyte (Tenzer et al., 2011; Schöttler et al., 2016). Fibrinogen another group of plasma proteins activate proinflammatory pathways and it is involved in the formation of blood clot (Deng et al., 2011; Monopoli et al., 2011a).

Immunoglobulins is another major component of plasma protein which play a key role in the immune system. This type of protein is consisting of five isotypes as follows, IgG, IgA, IgM, IgD, IgE. The smallest isotype of immunoglobulins, IgG is the only antibody that is able to across the placenta, and the biggest isotype IgM is the first antibody
that react to infection (Sacchetti et al., 2013; Gunawan et al., 2014). Aggarwal et al has been reported that apolipoprotein, complement proteins, fibrinogen and immunoglobulins exist in the protein corona of almost any NP (Aggarwal et al., 2009).

Adsorption of certain subset of proteins on the surface of nanoparticles enhance the uptake by macrophages cells of the reticuloendothelial system (RES). This category of proteins which is called opsonins is included of IgG, complement factors, and fibrinogen. Binding of opsonins to nanoparticles make a “molecular signature” for immune system, causing clearance from blood circulation and accumulation in the liver and spleen (Aggarwal et al., 2009; Walkey and Chan, 2012; Pearson et al., 2014b; Lee et al., 2015).

Karmali and Simberg reviewed the identity of corona profile on different nanoparticles. They have concluded that on liposomes and polymeric nanoparticles, apolipoproteins are the main group of proteins that adsorb on the surface of nanoparticles, but this does not imply for inorganic nanoparticles. By reducing hydrophobicity of polymeric nanoparticles, the adsorption of ApoA-I, ApoA-IV, ApoC-III and ApoJ was decreased, while there was no change in the absorption level of IgG and albumin. Albumin had strong affinity for cationic lipoplexes and polyplexes as well as hydrophobic surfaces. The protein profile on hydrophilic inorganic nanoparticles significantly differ than polymeric nanoparticles with hydrophobic surface. Transferrin, haptoglobin, fetuin A, kininogen, histidine-rich glycoprotein, and clotting pathway factors were found on hydrophilic inorganic nanoparticles. Absorption of complement C3 was increased by presence of hydroxyl group on nanoparticle surface (Karmali and Simberg, 2011).
2.2.2 Mechanisms and kinetics of protein adsorption

Protein corona forms through a dynamic process and adsorbed proteins are in constant state of flux. More specifically, protein corona is not a fix layer and proteins on NP surface are in continues exchange with free proteins in biological medium (Aggarwal et al., 2009; Rahman et al., 2013; Kharazian et al., 2016). Formation of protein corona is a time dependent process which it evolves by the time. In particular, the composition of protein corona evolve considerably from what was formed at the initial stages due to ongoing exchange of high abundance protein which adsorb first with low abundance proteins adsorbing to nanoparticles surface afterward (Saptarshi et al., 2013; Gunawan et al., 2014; Wan et al., 2015; Corbo et al., 2016; Vilanova et al., 2016).

The time evolution of protein corona formed around gold nanoparticles (AuNPs) in the cell culture media with 10% fetal bovine serum (FBS) was studied. The size of AuNPs were in the range of 4 to 40 nm and were stabilized with citrate ions, self-assembled monolayer (SAM) of mercaptoundecanoic acid (negative surface charge) and self-assembled monolayer (SAM) of aminoundecanethiol (positive surface charge). By increasing the incubation time, an enhancement in the hydrodynamic diameter, decrease in the surface charge and the red-shift of surface plasmon resonance was observed. This result indicates that the protein corona was evolved from a loosely weakly bound protein toward an irreversible persistent protein corona over time (Casals et al., 2010).

The changes occurred over time in corona profile of lecithin-coated polystyrene nanosphere were investigated by Nagayama et al. In a liver perfusion study by employing
SDS-PAGE and western blotting the protein corona was analysed quantitatively and qualitatively in the time period of 5 to 360 minutes. Over time, an increment in the total amount of adsorbed proteins on nanoparticles surface was observed. Moreover, there were significant changes in the qualitative profile of protein in which complement C3 (C3) and immunoglobulin G (IgG) showed enhancement by the time and there was a slight increase in apolipoprotein E (ApoE) and immunoglobulin A (IgA). The hepatic uptake by liver macrophages (Kupffer cells) was higher over the time indicating increased opsonisation of NP (Nagayama et al., 2007).

Protein corona evolves also as NP migrate from one biological compartment to another. The final corona retains the memory of its journey within the body. Thus, the composition of protein corona depends on all the environments which NP has passed through (Milani et al., 2012; Monopoli et al., 2012; Schleh et al., 2012; Maiolo et al., 2015). This concept can be employed to track the biodistribution of NP which in turn is important in nanotherapeutics applications (Gunawan et al., 2014; Hamad-Schifferli, 2015; Schöttler et al., 2016).

Lundqvist et al. studied protein corona evolution following moving from one biological fluid to another. They have simulated in vivo transport by incubating silica, polystyrene, and carboxyl-modified polystyrene NPs in human plasma following incubating the NPs in cytosolic fluid. The result showed remarkable evolution of the protein corona over time but the final corona after second incubation, encompasses the “fingerprint” of its history. They suggested that this phenomenon can be utilized to trace
the transport pathway of nanoparticle as well as the fate and biological behaviour of NP 
(Lundqvist et al., 2011).

Recently, researchers are applying quantitative models to determine the 
associations between the structure of protein corona and distinctive protein corona ‘fingerprints’. Chan et al. established a novel model to predict the biological behaviour of nanoparticle. They applied fingerprint of protein corona formed around 105-member library of surface-modified gold nanoparticles. They concluded that this model was 50% more accurate than previous model which utilize physicochemical properties of nanoparticle such as size, surface charge and aggregation state (Walkey et al., 2014).

The rate of adsorption/desorption of proteins over time refers as kinetics of protein corona. Kinetics rate of each protein determine composition of protein corona at any given time. The possibility of the contact between nanoparticle-protein and probability of that contact lead to adsorption of protein defined by association constant \( (k_{on}) \). As such, dissociation constant \( (k_{off}) \) represent the binding energy of nanoparticle-protein complex, in which the higher the binding energy the lower the dissociation constant. The balance between association rates \( (k_{on}) \) and dissociation rate \( (k_{off}) \) of a protein is defined by binding constant \( (K_d) \) and indicates which proteins will be bound to the NP surface at equilibrium conditions. (Ehrenberg et al., 2009; Dell'Orco et al., 2010; Walkey and Chan, 2012; Mahmoudi et al., 2013a; Del Pino et al., 2014).
The dynamic process and evolution of proteins on the flat surface was analysed by Vroman at 1962 (Vroman, 1962). This researcher explained the complex series of proteins displacement by time known as “Vroman Effect” which has been applied to nano-surfaces as well. “Vroman Effect” states the identity of proteins adsorbed on the surface varies over time although, the total quantity of the adsorbed protein remains constant. (Jansch et al., 2012; Vogler, 2012; Docter et al., 2015a; Kharazian et al., 2016). This phenomenon which depends on the abundance and affinity of the proteins along with their diffusion coefficients., is consist of two distinct stages refereed as ‘early’ and ‘late’ stage. During the early stage, adsorption of albumin, IgG, and fibrinogen take place which are highly motile proteins. These proteins will be then replaced by more static proteins such as apolipoproteins and coagulation factors during the late stage (Walkey and Chan, 2012; Rahman et al., 2013). Kinetic study on solid lipid nanoparticles (SLN) showed that in the early stage albumin was adsorbed which was then replaced by fibrinogen. Over time IHRP (inter-α-trypsin inhibitor family heavy chain-related protein) and apolipoproteins substitute fibrinogen, indicating being in agreement with “Vroman effect” (Göppert and Müller, 2005).

Protein corona on nanoparticles are so thick to be considered as a monolayer of proteins but composed of multiple layers like Christmas tree structures (Walkey and Chan, 2012; Rahman et al., 2013; Docter et al., 2015a; Docter et al., 2015b). Protein corona can be classified into two different types of protein layers, an inner layer which is consist of tightly bound proteins that they don’t readily desorb, termed “hard corona” and an outer layer comprise the loosely bound proteins, referred as “soft corona”. Hard corona represents proteins with high affinity and low-abundance which are characterized by slow
exchange rate with the biological medium. In contrast, soft corona represents proteins with low affinity and high-abundance characterized by rapid exchange rate with the biological medium (Tenzer et al., 2013; Brun and Sicard–Roselli, 2014; Walkey et al., 2014; Westmeier et al., 2015; Zanganeh et al., 2016).

Soft and hard corona can also be defined based on their residence time. Hard corona proteins have long residence time and they are more stable while soft corona proteins have short residence time and are more dynamic (Lynch and Dawson, 2008; Mahmoudi et al., 2011a; Hadjidemetriou et al., 2015). Due to their long lifetime on NP, hard corona resides on NP surface and undergo more biological process such as endocytosis. As such, hard corona plays more important role in determining the physiological response than soft corona (Nel et al., 2009; Walczyk et al., 2010).

In a model proposed by Cedervall et al protein corona was distinguished as fast and slow components. Fast component was formed in seconds around NIPAM/BAM nanoparticles whereas slow component was adsorbed within hours. Desorption pattern also indicated the same trend with lifetime of roughly 10 minutes for the fast component and almost 8 hours for the slow component (Cedervall et al., 2007).

It is hypothesized that proteins in hard corona, interact directly with the NP surface, while proteins of the soft corona interact with proteins of hard corona through weak protein–protein interactions (Walkey and Chan, 2012; Polyak and Cordovez, 2016). A model has been suggested by Simberg et al in which protein corona is composed of “primary binders” and “secondary binders”. The former directly recognize nanoparticles
surface while the latter interact with the primary binders. The activity of primary binders might be altered by secondary binders as they are masked, leading to avoid interaction of primary binders with the biological medium (Simberg et al., 2009).

2.2.3 Biological consequences of protein-corona formation on nanoparticles

Protein corona may affect numerous physiological responses such as toxicity, uptake, and biodistribution of nanoparticles. Formation of protein corona can be beneficial or disadvantageous in biomedical application of nanoparticles. Knowledge of implications of protein corona in clinical application of nanoparticles is of crucial importance to design the safe and applicable nanoparticles. Understanding the effect of protein corona on physiological responses enable preventing binding of certain proteins which stimulate phagocytosis and decrease blood circulation time of nanoparticle-based therapy. In the other hand, can make use of protein corona by designing nanoparticles to adsorb proteins of interest for targeting purpose which help directing of nanoparticle to the site of interest (Helou et al., 2013; Sobczynski et al., 2014; Lazarovits et al., 2015; Mirshafiee et al., 2016b).

Even though identical nanoparticles in different studies were applied, contradictory outcomes in cytotoxicity have been reported. Protein corona can affect toxicity profiles of nanoparticles in different ways. More specifically, protein corona may reduce NP-induced toxicities by acting as an interface in interactions with cell membrane and preventing cell membrane rupture (Corbo et al., 2016). In absence of protein corona,
NP interact with cell membrane proteins directly and disrupt the integrity of cell membrane leading to cell death (Ruenraroensak et al., 2012; Wolfram et al., 2014).

The impact of fetal bovine serum (FBS) driven protein corona on toxicity profile of Graphene Oxide (GO) nanosheets was evaluated. The cytotoxicity study at different concentration of FBS (1% and 10%) revealed that at low concentration (1%) cytotoxicity was in a concentration-dependent manner whereas by increasing serum concentration to 10% the cytotoxicity of nanoparticles was highly reduced. Moreover, it was shown that the cytotoxicity of GO nanosheets occurred due to direct interaction of GO nanosheets with cell membrane thereby causing the cell membrane undergone severe damage (Hu et al., 2011b).

Due to negative charge of cell membrane the role of protein corona in reduction of toxicity can be more significant when NP is positively charged (Molinaro et al., 2013; Wang et al., 2013). Protein corona of positively charged polystyrene NPs was retained on nanoparticles surface as were taken up by cells and trafficked to lysosomes. In this compartment, corona coated NPs was degraded and lysosomal content was released. Hence, protein corona protected the cell from any damage caused by bare NPs till it was cleared through lysosome (Wang et al., 2013).

Toxicity of protein corona coated carbon nanotubes CNTs on human acute monocytic leukemia cell line (THP-1) and human umbilical vein endothelial cells (HUVECs) were examined by Ge et al. It was found that protein corona significantly reduced toxicity of CNTs and as the density of adsorbed proteins increased, toxicity of
CNTs decreased (Ge et al., 2011). Tenzer and co-workers studied the effect of protein corona on toxicity and pathophysiology of nanoparticles. It was found that pristine silica NPs triggered thrombocytes activation and caused hemolysis. whereas, protein corona formed on silica NPs inhibited these adverse effects and protected the cells from damage (Tenzer et al., 2013).

In addition to protecting cell membrane, formation of protein corona on nanoparticle increase their stability which in turn mitigate toxicity. This is prominent specially for those nanoparticles which are not stable like quantum dots and their degradation leads to release of toxic product (Corbo et al., 2016; Westmeier et al., 2016). Moreover, toxicity of NPs can be associated to formation of reactive oxygen species (ROS) when NPs have semiconductor features. In this case, formation of protein corona prevents generation of ROS and increase the safety of NPs (Manke et al., 2013; Minai et al., 2013). Cytotoxicity of cobalt oxide on human monocytic cell line (THP-1) was examined by Casals, 2011. It was observed that following incubation with serum, toxicity profile of cobalt oxide was remarkably reduced due to decrease in ROS generation (Casals et al., 2011). In another study, prior to exposure of ZnO NPs to human hepatocellular carcinoma (HepG2) cell, ZnO NPs pre incubated with cell culture medium. It was observed that the cytotoxicity of pre coated ZnO NPs were remarkably decreased to compare with pristine ZnO NPs. It was concluded that due to increase in amount and affinity of adsorbed proteins on NP surface, ROS formation as well as ZnO dissolution were inhibited leading to highly reduction of cytotoxicity (Yin et al., 2015).
On the contrary, NP may cause denaturation in adsorbed proteins which can trigger toxicity. For instance, poly(acrylic) acid conjugated gold nanoparticles caused unfolding in the bound fibrinogen which in turn activated inflammatory signalling pathways result in release of inflammatory cytokines (Deng et al., 2011). The correlation between formation of protein corona and cellular uptake has been established. The nature of adsorbed plasma proteins is a determinant factor in degree and rate of cellular uptake (Laurent et al., 2013; Lee et al., 2015; Maiolo et al., 2015; Westmeier et al., 2015). More specifically, a subset of plasma proteins called “opsonin” which includes immunoglobulins and complement proteins enhance the cellular uptake while another subset of plasma protein referred as “dysopsonins” such as albumin lowers the uptake level (Owens and Peppas, 2006; Moghimi et al., 2011; Walkey and Chan, 2012). In some cases, protein corona diminishes nanoparticles adherence to the cell membrane result in reduction of uptake (Lesniak et al., 2012; Smith et al., 2012; Wolfram et al., 2014).

The role of presence of protein corona on silica nanoparticles uptake by A549 lung epithelial cells was investigated. It was reported that the same nanoparticles show different biological responses depends on presence or absence of protein corona. In particular, in the absence of protein corona due to stronger adhesion of silica nanoparticles to the cell membrane higher internalization efficiency was observed (Lesniak et al., 2012). Oligonucleotide-functionalized gold nanoparticles (DNA-Au NPs or siRNA-Au NPs) were shown to have higher uptake in serum-free medium by HeLa cells. Pharmacological methods revealed that the serum proteins impaired the adhesion of nanoparticles to membrane of Hela cells (Patel et al., 2010).
The uptake of dihydrolipoic acid-coated quantum dots (DHLA-QDs) by HeLa cells were studied by Treuel and co-workers. It was observed that following formation of protein corona on nanoparticles surface the uptake level has substantially deceased (Treuel et al., 2014). Using carboxyl functionalized Iron platinum (FePt NPs), it was shown that after exposure to human transferrin the uptake of these nanoparticles by HeLa cells was highly reduced (Jiang et al., 2010). Wang et al. investigated uptake of Gold nanoparticles (AuNPs, 20 nm) by mouse myogenic (Sol8) cells in presence and absence of protein corona. It was shown that presence of protein corona supress the nanoparticles to be taken up by mouse myogenic cells (Wang et al., 2012).

On the contrary, some other researches indicated that protein corona facilitate uptake of particles by cells. The contradictory results may be due to different types of uptake that take place by cell such as specific or non-specific cellular uptake. Specific uptake is mediated by membrane receptors and have been reported to increase in presence of protein corona whereas, non-specific cellular uptake is regardless of cell receptors and is a random process which decrease with formation of protein corona (Brun and Sicard–Roselli, 2014; Schöttler et al., 2016). The impact of presence of protein corona on degree of specific uptake was assessed by Krais, et al, 2014. Uptake study of folic acid-functionalized iron oxide nanoparticles by ovarian cancer cells revealed that existence of protein corona is a requisite for uptake of nanoparticles (Krais et al., 2014). Using titanium dioxide (TiO₂) nanoparticles, uptake of these particles by A549 and H1299 human lung cell lines after incubation with fetal bovine serum (FBS) were evaluated. It was shown that formation of protein corona increased the level and rate of nanoparticles uptake (Tedja et al., 2012a).
In addition, employing different cell lines in uptake studies may demonstrate contrasting results. While protein corona supress uptake of nanoparticles by monocytes due to blocking the surface of nanoparticle, some cells such as macrophages, neutrophils, and dendritic cells express receptors on their surface that enable them to interact with opsonins in protein corona result in triggering internalization (Karmali and Simberg, 2011; Goodridge et al., 2012; Wolfram et al., 2014; Corbo et al., 2016). Effect of different cell lines on uptake of nanoparticles in presence of protein corona was investigated by Yan et al. Uptake of disulfide-stabilized poly (methacrylic acid) nanoporous polymer particles (PMA$_{SH}$ NPPs) by monocytes and macrophages was compared. Monocytic cells, THP-1 internalized nanoparticles much fewer following formation of protein corona than bare nanoparticles. Uptake of nanoparticles by differentiated macrophage-like cells (dTHP-1) has shown an increment compared to bare nanoparticles due to trigger of scavenger receptors (Yan et al., 2013).

2.3 Cellular uptake pathways of NPs

Cell membrane (CM) employs different mechanisms to exchange substances which are mainly divided into two categories: passive transport and active transport. Gases such as oxygen and carbon dioxide, hydrophobic molecules such as benzene and uncharged molecules such as water and ethanol diffuse across the membrane from the regions of higher to lower concentration. This kind of transport which is along the concentration gradient and occurs without assistance of energy is called passive transport. In contrast, active transport occurs against the concentration gradient by using energy which is provided by adenosine triphosphate (ATP) (Feher, 2012; Backes, 2015; Douglas
Polar or charged biomolecules that cannot pass through the hydrophobic plasma membrane are internalized by a form of active transport which is called endocytosis. In this process, the cell engulfs the materials inside the extracellular fluid by invagination of CM and buds off inside the cell, forming a membrane-bounded vesicle called an endosome (Makaraci and Kim, 2018).

Endocytosis can be basically classified into two major categories: phagocytosis and pinocytosis. Phagocytosis (cell eating) is the process of taking in debris, bacteria or other large size solutes by specialized mammalian cells called phagocytes (i.e. monocytes, macrophages and neutrophils) (Nazario-Toole and Wu, 2017; Rajendran et al., 2018). Integral to phagocytosis is a process called opsonization by which opsonins such as immunoglobulins and complement proteins coat the target materials to trigger the phagocytes of their presence and to initialize phagocytic activity (Xiang et al., 2012). As the phagocyte begins to ingest the target material, it will simultaneously stimulate the formation of a membrane-bound vesicle called phagosome into which the ingested materials are compartmentalized within the phagocyte. At the latter stages of this process, the phagosome will fuse with the lysosome and the materials are digested at acidic pH by the hydrolytic enzymes contained within the lysosomal lumen (Hillaireau and Couvreur, 2009; Xiang et al., 2012).

In all cell types, small particles within the range of nanometers are internalized by pinocytosis (Zhao et al., 2011). In pinocytosis, “cellular drinking” plasma membrane
forms an invagination to take up a small droplet of extracellular fluid including dissolved molecules in it. Pinocytosis is not a discriminating process and it occurs in almost all the cells in a continuous manner irrespective to the needs of the cell. The grabbed substances are pinched off into small vesicles that are called pinosome which fuses with lysosomes to hydrolyse or break down the contents (Ramsden, 2018).

Phagocytosis and pinocytosis can be distinguished by the size of their endocytotic vesicles; the former encompass uptake of large particles by large vesicles with the size of 250 nm, and the latter encompass uptake of fluids through small vesicles with the size in the range of a few nanometres to hundreds of nanometres (Panariti et al., 2012). Pinocytosis can be subcategorized into clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin- and caveolae-independent endocytosis and macropinocytosis (Hirota and Terada, 2012; Yu, 2018). Clathrin-mediated endocytosis is the cellular entry mechanism to internalize specific molecules into the cells. This entry route aids cells to take in plasma membrane components and nutrients including cholesterol by low density lipoprotein receptor and iron by transferrin receptor (Ferguson et al., 2017; Hassinger et al., 2017).

In this process, particular ligands in extracellular fluid bind to the receptors on the surface of the CM forming a ligand-receptor complex. This ligand-receptor complex moves to a specialized region of the CM which are rich in clathrin, whereby they are engulfed through the formation of clathrin-coated vesicles. Once inside the cell, clathrin
coatings on the exterior of the vesicles are expelled prior to fusing with early endosomes. The cargo within early endosomes will eventually reach lysosomes via the endo-lysosomal pathway (Rappoport, 2008; Cocucci et al., 2012).

Each type of NP is internalized by the cell via preferentially uptake pathway. For example, NPs composed of poly (lactic-co-glycolic acid), D,L-polylactide and poly(ethylene glycolco-lactide) and silica (SiO2)-based nanomaterials are internalized by clathrin-mediated endocytotic pathway (Sahay et al., 2010). Coumarin-based solid-lipid NPs are internalized by the cells via non-energy-dependent pathway as the structure of these NPs are similar to the CM. All the lipid-based NPs utilize the clathrin-mediated endocytosis pathway (Rivolta et al., 2011). The herceptin-coated gold NPs enter the cell via receptor-mediated endocytosis by means of membrane ErbB2 receptor (Rivolta et al., 2011).

Caveolae-mediated endocytosis is the route of cellular entry which involves flask-shaped membrane invaginations called caveolae (little caves). Caveolae are present in endothelial cells, epithelial, adipocytes, muscle and fibroblasts cells (Parton and Simons, 2007; Wang et al., 2011). The size of caveolae typically ranges from 50 to 80 nm and are composed of membrane protein caveolin-1 which confer them flask-shaped structure (Ray and Mitra, 2017). Caveolae-dependent endocytosis is involved in cell signaling and regulation of membrane proteins, lipids and fatty acids (Nabi, 2009). Once caveolae are detached from plasma membrane, they fuse with a cell compartment called caveosomes
that exists at neutral pH. Caveosomes are able to bypass lysosomes and therefore protect the contents from hydrolytic enzyme and lysosomal degradation. Hence, pathogens including virus and bacteria use this entry route to prevent degradation. Since the cargo internalized into the cells by caveolin-dependent mechanism do not end up in the lysosome, this pathway is employed in nanomedicine (Oh et al., 2007; Sandvig et al., 2011).

Clathrin- and caveolae-independent endocytosis occurs in cells that are deprived of clathrin and caveolin. This pathway is utilized by growth hormones, extracellular fluid, glycosylphosphatidylinositol (GPI)-linked proteins and interleukin-2 to enter the cells (Mellman and Nelson, 2008). For instance, folic acid that employs clathrin- and caveolae-independent pathway to enter the cells are conjugated to NPs and polymers used in drug delivery systems and as imaging agents (Lu and Low, 2012). Macropinocytosis is a type of pinocytosis mechanism in which cells take in high volumes of extracellular fluid by forming a large vesicle (0.5–10 μm) called macropinosomes (Mercer and Helenius, 2009; Lim and Gleeson, 2011). Macropinocytosis is a pathway to internalize apoptotic and necrotic cells, bacteria and viruses as well as antigen presentation. This pathway can internalize micron-sized NPs which are not possible to be taken into cells by most other pathways. Macropinocytosis can occur in almost any cells except for brain microvessel endothelial cells (Kerr and Teasdale, 2009; Kühn et al., 2017). NPs enter into the cell via one of these endocytotic routes as depicted in Figure 2.2.
Figure 2.2: Entry of NPs into cell using different endocytotic pathways. A) Macropinocytosis and phagocytosis. B) Clathrin-mediated endocytosis, clathrin-caveolin independent endocytosis and caveolae-mediated endocytosis.
2.4 Cellular ageing and senescence

2.4.1 Characteristic features of senescence

Throughout life cells encounter exogenous and endogenous stress and damage. Depends on the level and nature of stress as well as cell type, their responses vary from repair to cell death. Somatic cells can develop an additional response which is locking the cell into cell cycle leading to irreversible arrest of proliferation that is called cellular senescence. Therefore, senescence prevents the damage to be transmitted to the next cell generation (Campisi and di Fagagna, 2007; Kuilman et al., 2010; Sabin and Anderson, 2011).

Cellular senescence is characterized by a flat and enlarged morphology, irreversible cell cycle arrest and resistance to apoptosis (Figure 2.3). Senescence cells show dramatic changes in gene expression and chromatin organization. In addition, they express the senescence associated- $\beta$-galactosidase (SA-$\beta$-gal) (Chen et al., 2007; de Jesus and Blasco, 2012). By means of senescence biomarkers it has been shown that senescent cells accumulate in tissues with age, making the senescence as an excellent in vitro model for ageing studies (Debacq-Chainiaux et al., 2016; Wadhwa et al., 2016). Cellular senescence based on the nature of stimuli can be categorized to telomere dependent senescence and nontelomeric senescence termed replicative senescence and stress induced premature senescence, respectively (Zhao and Darzynkiewicz, 2013; Debacq-Chainiaux et al., 2016).
Figure 2.3: Features of senescent cells: Several markers were identified to characterize the senescent state in relation to morphology and proteostasis. Adapted from (Höhn et al., 2017).

2.4.2 Replicative senescence

Hayflick and Moorhead in 1961, (Hayflick and Moorhead, 1961) observed that despite the accessibility of adequate growth factors, nutrients, and space, human diploid fibroblasts (HDFs) have finite proliferation potential. These cells after a determined number of duplications stop dividing in culture. This phenomenon is called replicative senescence (RS) since it occurs by replication (Adams, 2009; Kuilman et al., 2010; Becker
and Haferkamp, 2013; Victorelli and Passos, 2016). Prior reaching the end of their replicative life span, the number of replication that cells undergo is termed Hayflick limit (Campisi, 2013; Triana-Martinez et al., 2016).

Cell type and the age of the donor are determinant factors in the number of population doublings (PDs) that normal cell cultures can achieve before senescence and it is not correlated to the culture’s chronological age (Toussaint et al., 2000; Maier and Westendorp, 2009; Wadhwa et al., 2016). There are some cells that they have never reach replicative senescence such as embryonic germ cells and tumour cells, called immortal cells (de Magalhaes, 2004). Later in 1990 Harley et al demonstrated that replicative senescence is associated to telomere length. (Harley et al., 1990). Telomeres are sequences of repetitive DNA that cover the end of chromosome in order to protect DNA from degradation or recombination and consequently keep the genomic stability (Campisi and di Fagagna, 2007; Collado et al., 2007; de Jesus and Blasco, 2012).

Due to inability of DNA polymerases to replicate DNA ends, telomere is eroded with every round of cell replication, so called “the end-replication problem”. This phenomenon cause the telomere reach the critical length and be dysfunctional which trigger DNA damage response (DDR). The DDR signals allows the cells to identify damaged DNA as double-strand breaks (DSBs), and consequently arrest cell-cycle progression and induce senescence (Maritz et al., 2013; Lasry and Ben-Neriah, 2015; Victorelli and Passos, 2016). An enzyme called telomerase circumvent the end-replication problem. Telomerase compensate telomere length by adding the repetitive telomeric DNA to chromosome ends therefore maintain telomere function (Hornsby, 2011; Childs et al.,
Telomerase is not expressed in human somatic cells in contrast to stem cells and germ cells which maintenance of telomeres is essential for genomic integrity and cell viability (Sabin and Anderson, 2011; Itahana et al., 2013).

2.4.3 Stress induced premature senescence

Beside telomere erosion, there are a range of nontelomeric stimuli that can cause senescence prematurely in cells. “Premature” states that senescence arises prior to maximum number of population doublings at which replicative senescence occurs, in the other word, “premature” show the quickening of the process (Kuilman et al., 2010; Debacq-Chainiaux et al., 2016). The term “stress-induced premature senescence” is referred for senescence subsequent from all types of cellular stress except telomere dysfunction (Toussaint et al., 2000; Hornsby, 2010). These stresses can be caused by internal or external, physical or chemical, acute or chronic agents. Stress-induced premature senescence comprise oxidative stress as a consequence of mitochondrial deterioration, genotoxic stress resulting from disrupted chromatin and oncogenic stress leading to oncogene-induced senescence (Chen et al., 2007; Davalos et al., 2010; Loaiza and Demaria, 2016).

2.4.3(a) Oxidative stress

Oxidative stress is the most common cause of stress-induced premature senescence. Oxidative stress, caused by increased concentration of reactive oxygen species (ROS) in the cell, can happen as a consequence of mitochondrial dysfunction induced by the oncogenic RAS (Moiseeva et al., 2009) or independent of oncogenic
signaling. Various oxidative stresses are utilized to trigger premature senescence, (Passos et al., 2007; Vurusaner et al., 2012; Ale-Agha et al., 2014; Klement and Goodarzi, 2014) such as exposure to hydrogen peroxide (Chen, 2000), ultraviolet (UV) light (Ma et al., 2002; Wlaschek et al., 2003; Debacq-Chainiaux et al., 2005), tert-butylhydroperoxide (Dumont et al., 2000b) and hyperoxia (Dumont et al., 2000a).

In addition to the mentioned factors that cause premature senescence, in vitro culture condition will also result in inducing premature senescence. Difference in the ambient of the cells such as difference in O$_2$ levels, abnormal concentrations of nutrients and growth factors make the in vitro culture condition as an artificial, harsh and stressful environment for the cells deprived of adjacent cells and extracellular matrix components. Any of these conditions enforce culture shock leading to stress induced premature senescence (Sherr and DePinho, 2000; Rodier and Campisi, 2011).

2.4.3(b) Genotoxic stress

A cell's genomic integrity is at risk when DNA-damaging stress, evoked by mitogenic oncogenes or genotoxic treatment modalities such as radiation or chemotherapy, apply. If the DNA repair machinery fails to fix the damaged site during a temporary cell-cycle arrest, or if massive genotoxic stress overwhelmed the repair capacity, cellular failsafe programs such as apoptosis or senescence will be triggered to limit aberrant propagation of these damaged and potentially harmful cells.
Genotoxic stress which is caused by disrupted chromatin may be induced by cisplatin, cyclophosphamide, doxorubicine, taxol, vincristine, cytarabine, etoposide, hydroxyurea, bromodeoxyuridine and adriamycin (Becker and Haferkamp, 2013; Piegari et al., 2013; Loaiza and Demaria, 2016).

2.4.3(c) Oncogene-induced senescence

Another nontelomeric form of stimulus that may cause senescence in cells include over activation of oncogenes. Developing senescence is a response to oncogenes in which cells undergoing through. This form of senescent was first observed at 1997 by Serrano et al (Serrano et al., 1997) in human fibroblasts upon expression of oncogenic version of RAS which is a cytoplasmic transducer of mitogenic signals. Since then this phenomenon was called oncogene-induced senescence (OIS). Subsequently, it was shown that overexpression of any cellular pathway contributing in proliferation may lead to senescence (Campisi and di Fagagna, 2007; de Jesus and Blasco, 2012; Lasry and Ben-Neriah, 2015).

2.4.4 Biomarkers of cellular senescence

As a result of changes in cell and organelle size that cell experience during senescence and also changes in cell function, senescent cells exhibit characteristic that lead to identify these cells in vitro and in vivo. Nevertheless, no marker is exclusive to the senescent phenotype, additionally, not all senescent cells express all the identified senescence markers. Therefore, senescent cells are commonly identified by series of
characteristics. These characteristic are explained in the following sections (Young et al., 2013; Nehlin, 2016).

2.4.4(a) Growth arrest

An indispensable marker of cellular senescence is an inability of DNA replication which can be detected by the incorporation of BrdU (5-bromodeoxyuridine) or 3H-thymidine. As opposed to quiescence, growth arrest in senescence is permanent and cannot be stimulated by any physiological stimuli. Senescent cells are arrested at G1/S phase of the cell cycle, yet they remain metabolically active (Herbig et al., 2004; Campisi, 2011; Carnero, 2013).

2.4.4(b) Morphology

Cells undergoing senescence show striking changes in morphological feature. Senescent cells are enlarged with multiple nuclei. Golgi apparatus and cytoplasm of senescent cells turn to be prominent and vacuolated, respectively. In addition, senescent adherent cells appear flattened, muddled and are arbitrarily oriented in culture dish (Kuilman et al., 2010; Becker and Haferkamp, 2013; Wadhwa et al., 2016).

2.4.4(c) Apoptosis resistance

Senescent cells are extremely resistant to apoptotic death signal. This characteristic might explain the stability of senescent cells in culture as well as an
increment in number of senescent cells with age (Sasaki et al., 2001; Campisi and di Fagagna, 2007).

2.4.4(d) Gene expression

During senescence extreme alteration occur in gene expression profile especially genes related to cell cycle inhibitors and cell cycle activators. Cell cycle inhibitors, cyclin-dependent kinase inhibitors (CDKIs) p21 (CDKN1a) and p16 (p16INK4a) overexpressed and those genes that encode proteins to stimulate cell cycle are repressed (Zhao and Darzynkiewicz, 2013; Debacq-Chainiaux et al., 2016). In young cells cyclin-dependent kinase inhibitors are absence thereby expression of these genes is a common marker for detection of senescent cells (Campisi, 2013; Loaiza and Demaria, 2016).

2.4.4(e) Senescence associated-β-galactosidase

In 1995, Dimiri et al. (Dimri et al., 1995) discovered that in addition to lysosomal β-galactosidase which is detectable at pH 4, senescent cells expressed β-galactosidase, that is detectable at pH 6.0 called senescence-associated β-galactosidase. Since then, it has been the most extensively used biomarker to identify senescent cells in vitro and in vivo. This behaviour of β-galactosidase enzyme was shown is due to an increase in the number and size of lysosome and consequently elevated lysosome content in senescent cell (Kurz et al., 2000a; Lee et al., 2006; Maier et al., 2007; Itahana et al., 2013). β-galactosidase activity can be detected by histochemical staining of the cell by the chromogenic substrate
5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Lee et al., 2006; Debacq-Chainiaux et al., 2009; Carnero, 2013).

### 2.4.4(f) Senescence-associated heterochromatin foci

As the hallmark of senescent cells is irreversible proliferation arrest, once senescence occurs, proliferation-promoting genes such as E2F target genes form transcriptionally silent chromatin. Upon senescence, altered chromatin structure by means of generation and accumulation of distinct heterochromatic structures, called senescence-associated heterochromatin foci (SAHF). This characteristic of senescent cells was discovered by Narita et al. in 2003 who observed that the DNA of senescent cells comprise punctate domains of heterochromatin which can be readily differentiated from chromatin in nonsenescence cells. DNA dyes, such as 4′,6-diamidino-2-phenylindole (DAPI) can be applied to detect the SAHF since in nonsenescence cells DNA dyes show homogenous staining patterns whereas in senescent cell they show punctate staining patterns (Aird and Zhang, 2013; Nehlin, 2016).

### 2.4.4(g) Senescence-associated secretory phenotype

In spite of their growth arrest senescent cells are metabolically active and affect their microenvironment. Cells experiencing senescent exhibit significant changes in their secretome including expression of variety of proteins such as cytokines, chemokines, and proteases. This phenotype enables the senescent cells to communicate with neighbouring cells, called senescence-associated secretory phenotype (SASP) or senescence-messaging
secretome (SMS) (Kuilman et al., 2010; Acosta et al., 2013; Lasry and Ben-Neriah, 2015).

2.5 Cell death

Cell death is the event of a biological cell ceasing to carry out its functions. This may be the result of the natural process of old cells dying and being replaced by new ones, or may result from such factors as disease, localized injury, or the death of the organism of which the cells are part (Ianzini and Mackey, 2007; Kierszenbaum and Tres, 2015). In multicellular organisms, cell death is a critical and active process that maintains tissue homeostasis and eliminates potentially harmful cells (Bhattacharya and Ghosh, 2014). Cell death has a crucial role in our bodies during development and in regulating tissue homeostasis by eliminating unwanted cells (Fuchs and Steller, 2011). There are three major types of morphologically distinct cell death: apoptosis (type I cell death), autophagic cell death (type II), and necrosis (type III).

2.5.1 Apoptosis

Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms (Clarke and Clarke, 2012). In evolutionarily high animals, many cells are no longer needed during or after certain developmental or physiological states, such as during digit individualization in the human embryo, post-pubertal involution of the thymus, postpartum involution of the uterus, postlactating (post-weaning) involution
of mammary glands, etc., as some of us have reviewed before (Rezzani et al., 2014). Apoptosis is the mechanism used by the animal to get rid of these cells that are no-longer useful to it and thus are redundant. The animal requests these obsolete cells to die via a pre-determined procedure, or a “program”, thus making this death mode regarded as a “programmed cell death”. (Green and Llambi, 2015; Liu et al., 2018). One hallmark of apoptosis is that the cell death is physiological and should not cause any harm to the host tissue and certainly not to the entire organism (Galluzzi et al., 2015).

Apoptosis (type I cell death) occurs through a sequence of specific morphological changes in the dying cell: condensation of the cytoplasm and margination of the nuclear chromatin into one or several large masses, with subsequent formation of membrane-bound apoptotic bodies, containing a variety of cytoplasmic organelles and nuclear fragments, which are engulfed by neighboring cells and by macrophages (Manning and Zuzel, 2003; Elmore, 2007)

2.5.2 Autophagy

Autophagy can be described as a process of cell recycling through degradation by lysosomes. It involves intracellular membrane reorganization to create auto-phagosomes, which sequester cytoplasm and organelles. After that they fuse with lysosomes and the cargo is degraded and recycled (Kelekar 2005; Hoyer-Hansen et al. 2008).
Autophagy is generally activated by conditions of nutrient deprivation but has also been associated with physiological as well as pathological processes such as development, differentiation, neurodegenerative diseases, stress, infection and cancer (Codogno et al., 2012; Feng et al., 2013). In disease, autophagy has been seen as an adaptive response to stress, promoting survival of the cell; but in other cases it appears to promote cell death and morbidity. In the extreme case of starvation, the breakdown of cellular components promotes cellular survival by maintaining cellular energy levels (Papinski and Kraft, 2014; Schneider and Cuervo, 2014).

Autophagic cell death is characterized by the appearance of large intracellular vesicles and engagement of the autophagy machinery (Kroemer and Levine, 2008). Autophagy is mainly a survival process engaged in response to a metabolic crisis (e.g., low ATP levels and nutrient and amino acid deprivation) or to remove damaged organelles (e.g., mitochondria with low membrane potential) and protein aggregates (Galluzzi et al., 2018). As a stress response, autophagy accompanies rather than promotes cell death in most scenarios and merely represents a failed survival attempt (Shen et al. 2012).

2.5.3 Necrosis

Necrosis is a form of cell injury which results in the premature death of cells in living tissue by autolysis (Proskuryakov et al., 2003). Necrosis is cell death where a cell has been badly damaged through external forces such as trauma or infection and occurs in several different forms. In necrosis, a cell undergoes swelling, followed by uncontrolled
rupture of the cell membrane with cell contents being expelled. These cell contents often then go on to cause inflammation in nearby cells (Fayaz et al., 2014; Kers et al., 2016).

Necrosis is characterized by swelling of the endoplasmic reticulum, mitochondria, and the cytoplasm, with subsequent collapse of the plasma membrane and lysis of the cells. The term oncosis was proposed to refer to the early stage of primary necrosis, during which cells committed to death pass through a pre-lethal process in which they swell (Aki et al., 2015)
CHAPTER THREE: PREPARATION AND CHARACTERIZATION OF NANOPARTICLE-PROTEIN CORONA

3.1 Introduction

In this study, commercially available PEGylated non-targeted quantum dots (Qtracker® 705) were used to prepare hard protein corona coated nanoparticles. Hard protein coronas are referred to as proteins that adsorb to the nanoparticles irreversibly with high affinity and have a small disassociation constant. The high degree of stability and robustness makes hard protein corona a reliable candidate to be used in the current study. To simulate blood plasma level at human physiological conditions, the quantum dots were incubated in pooled human plasma at concentration of 55% (Anderson and Anderson, 2002). After performing a series of washing and centrifugation steps, hard corona coated quantum dots were characterized by various analytical methods to elucidate the newly formed structures and its monomer protein components.
3.2 Materials and methods

3.2.1 Nanoparticle preparation

The nanoparticles used in the experiments, Qtracker® 705 non-targeted quantum dots (QDs) were purchased from Invitrogen, Inc. Qtracker® 705 nontargeted quantum dots are composed of Cd/Se/Te core, covered with a ZnS shell and modified with methoxy-PEG-5000 coating. The human plasma used in this experiment were obtained from Sigma-Aldrich. The plasma was prepared from pooled human blood and it contains 4% trisodium citrate as an anticoagulant. Human plasma at an approximate concentration of 55%, which represents the average volume proportion of plasma in blood was used to prepare the hard protein corona throughout the experiment.

QDs with concentrations of 10-50 nM were prepared in PBS (100 µL) and added to 55% human plasma (900 µL). The solutions were mixed by repeated agitation in 2 mL microfuge tubes to obtain a final volume of 1 mL. The QDs incubated in the plasma solution were incubated at 37 °C for 1 h in a thermomixer. Immediately after incubation time has been completed, the solution in microfuge tubes were centrifuged at 14,000 rpm in a temperature controlled centrifuge at 15 °C for 40 minutes to pellet QD-protein corona complex. Supernatant were removed gently without disturbing the pellet and the QD-protein corona complex was resuspended 1 mL of cold PBS by mixing with a pipette until the solution is homogenous. The solution was then transferred to a new microfuge tube to prevent transfer of proteins adhered to the microfuge tubes back into the solutions and centrifuged again for 20 minutes at 14,000 rpm at 15 °C. The washing step is repeated one more time to obtain QD-hard corona complex.
3.2.2 Characterization of nanoparticles

3.2.2(a) Transmission electron microscopy (TEM)

Primary diameter of QD nanoparticle were characterized using transmission electron microscopy (TEM) (Model FEI CM12 version 3.2 image analysis systems 120kV) and high resolution transmission electron microscopy (HRTEM) (200kV with field emission, TECNAI G2 F20 S-TWIN, FEI). The Images from TEM and HRTEM was analyzed using ImageJ software whereby 100 particles were measured per sample.

3.2.2(b) Scanning transmission electron microscope in tandem with energy dispersive x-ray spectroscopy (STEM-EDX)

Elemental mapping and elemental/chemical characterization of the QDs were evaluated by scanning transmission electron microscope in tandem with energy-dispersive x-ray spectroscopy (STEM-EDX) (TECNAI G2 F20 S-TWIN, FEI).

3.2.2(c) Dynamic light scattering (DLS) and zeta potential measurement

Hydrodynamic diameter, size distribution and surface charge of QD nanoparticles were characterized using zetasizer (Model ZEN 3600, Nanoseries, Malvern Instruments). Dynamic laser light scattering measurements were checked using the single-scattering regime with a 633 nm He-Ne laser light source and a fixed detector angle of 173°. The temperature of the samples was automatically kept at 25°C. A low volume (200 µl) quartz crystal measuring cell was used. Prior to each experiment, the measurement cell was
flushed with isopropyl alcohol and then high purity water to remove any contaminants. The cell was then filled with the QD solution, and the data were measured and analyzed. Hydrodynamic diameter values were determined by averaging the intensity-weighted size distributions obtained from 20 successive DLS runs of 10s each. Measurements were repeated three-times and results are reported as mean ± standard deviation (SD). The distribution widths were quantified by the Polydispersity Index (PdI). The zeta potential value was determined by measuring the electrophoretic mobility of the QDs under the influence of an external oscillating electrical field with a voltage of 100V. The measured electrophoretic motilities were converted to zeta potentials by the instrument software using Henry’s equation. QD solution was transferred to a disposable zeta cell and measurements were carried out at 25°C after 120 s equilibration time, performing seven runs comprising 15 subruns each.

3.2.2(d) Atomic force microscopy (AFM)

22 X 22 mm glass cover slips were placed on the bottom of 33-mm wells and QDs nanoparticles prepared in culture medium was dispensed gently onto the wells. The well plates were incubated at 37°C for 30 and 90 minutes in a CO₂ incubator. After incubation, the culture medium was removed gently from the wells and the glass cover slips were rinsed gently with deionized water and dried with pressurized nitrogen prior to AFM analysis. Topographical scanning of QD sediments in dry conditions were performed on XE-BIO AFM (Park Systems) operating in non-contact mode. Silicon nitride AFM tips with a curvature radius of 2 nm and a nominal spring constant of 42 N/m were used to
probe the sediment heights. Ten images were acquired per substrate with a scan area of 10 X 10 μm$^2$ or 30 X 30 μm$^2$ depending on the nanoparticle distribution.

3.2.2(e) Fluorescence spectrometry

QD nanoparticles were incubated in solutions at cell cytosolic and lysosomal pH for 4 h. QD nanoparticles were incubated in ALF solution to simulate lysosomal pH at 4.5–5.0 and prepared as described by Stopford et al. (2003). Proteolysis of QD-HC nanoparticles were simulated using Proteinase K enzymes in ALF solution. QD nanoparticles were incubated in PBS to simulate cytoplasmic pH at 7. Photostability of QD nanoparticles were measured using fluorescence spectrophotometer at excitation/emission wavelength of 488/660-735 nm.

3.2.3 Characterization of hard protein corona

3.2.3(a) Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Hard protein corona was eluted from the nanoparticles by adding SDS sample buffer (62.5 mM Tris-HCl pH 6.8; 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) to the pellet obtained after the final washing step and incubated at 95 °C for 5 min. After incubation, the eluted proteins were separated using 15% SDS-PAGE and Mini Protean II electrophoresis cell from Bio-Rad at 120 V for about 55 min. the separated proteins on the gels were visualized by staining with Coomassie brilliant blue R-250 staining or silver staining. Coomassie Brilliant Blue staining were performed by
fixing the gels in staining solution (50% methanol/7% acetic acid, 0.1% Coomassie R-250 in 50% methanol/1% acetic acid) for 1 h and destained overnight in 30% methanol/1% acetic acid.

Silver staining was performed according to the manufacturer’s staining protocol from Bio-Rad. Fixation solution (50% methanol, 10% acetic acid, 10% fixative enhancer concentrate) was added on the gels and incubated for 20 minutes with gentle shaking. After incubation, the gels were rinsed twice with deionized distilled water for 10 minutes with gentle agitation. Then, the gels were stained with staining solution (50% development accelerator solution, 5% silver complex solution, 5% reduction moderator solution, 5% image development reagent) for 20 minutes. After the desired staining intensity was reached, the gels were placed in 5% acetic acid solution for 15 minutes to stop the staining reaction. Finally, the gels were rinsed in deionized distilled water and dried before imaging. Experiments were conducted twice to ensure reproducibility of the results.

3.2.3(b) Micro bicinchoninic colorimetric (µBCA) assay

µBCA assay was performed to estimate the protein corona that were adsorbed onto the NPs. BCA working reagents (copper solution) were mixed according to manufacturer’s recommendation of reagents A, B, and C in the ratio 25:24:1. The working reagents were then dispensed into 96-well microplates at a volume of 200µl per well. Different concentrations of BSA standards (0-25 µg/ml) were prepared in PBS solution to plot standard curve of Concentration versus Absorbance. The standard curve will be used to calculate protein concentration in unknown samples. BSA standards were added to
the previously prepared BCA working reagents at a volume of 50µl per well. Concurrently, protein corona samples extracted from different concentrations of NPs were added in the BCA working reagents at a volume of 50µl per well. The microplates were then incubated at 37 ºC for 2 hours. After the incubation period, the absorbance of samples was measured at 562 nm using spectrophotometer and the absorbance directly correlates to the concentration of protein corona. The assays were performed in three replicates and three independent experiments.

3.2.3(c) Matrix assisted laser desorption/ionization–time of flight (MALDI-TOF/TOF) mass spectrometry

Eluted proteins from QD nanoparticles were separated using 15% SDS-PAGE and the separated proteins were excised from the gels. To perform MALDI-TOF/TOF analysis, the samples were outsourced to Institute for Research in Molecular Medicine (INFORMM), USM. The samples were washed with ammonium bicarbonate, treated with dithiothreitol and subsequently digested with trypsin before analysis with MALDI-TOF/TOF 5800 mass spectrometry. Peptide mass spectra with the highest intensity were compared against protein sequence database (uniprot) using the MASCOT protein identification program (Matrix Science, Ltd.).

3.2.3(d) Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Hard protein corona was eluted from the nanoparticles by adding SDS sample buffer (62.5 mM Tris-HCl pH 6.8; 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v
bromophenol blue) to the pellet obtained after the final washing step and incubated at 95 °C for 5 min. After incubation, the eluted proteins were collected and transferred into new microfuge tubes. To perform LC-MS/MS analysis, the samples were outsourced to Analytical Biochemistry Research Centre (ABrC), USM. Buffer exchange, in solution trypsin digestion and peptide concentration were performed on the samples before LC-MS/MS (LTQ-Orbitrap) analysis. De novo sequencing and database matching (SwisProt) was performed using PEAKS Client Version 7.5 (Bioinformatics Solution).

3.2.4 Statistical analyses

Statistical analysis was performed based on one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer post hoc test of significance by using Prism® 5.0 software (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered to be statistically significant.
3.3 Results and Discussion

3.3.1 Size, morphology and elemental analysis of nanoparticles

Morphology of QD-PEG NPs were determined by transmission electron microscopy (TEM). Based on TEM micrographs, pyramidal shaped structures with an average side length of 10 ± 1.5 nm characterizes most of the nanoparticles (Figure 3.1A). To obtain greater resolution on the structure of isolated pyramidal nanoparticles and plausibly measure the thickness of polyethylene glycol (PEG) coatings on the outermost surface of nanoparticles in desiccated state, high-resolution transmission electron microscopy (HRTEM) was employed. Within the architecture of QD-PEG NP system, amphiphilic polymer coating PEG was incorporated to increase the dispersibility of NPs in biological mediums while concurrently reducing its agglomeration (Ulusoy et al., 2015; Guerrini et al., 2018).

The surface characteristics of the nanoparticle is vital in this experiment considering its influence in determining the type and the amount of protein adsorption on the nanoparticles during hard corona preparation. The surface modification will in turn affect the interaction of the nanoparticles with cells such as cytotoxicity and cell uptake characteristics (Mahmoud et al., 2010; Zhao et al., 2011). The HRTEM image of the pyramidal nanoparticles displayed several lattice planes of electron dense core/shell structure but the PEG layers were not spotted (Figure 3.1E). The PEG layer and the surface of the copper grid on which the nanoparticles were deposited are both amorphous in nature, hence rendering it to be indistinguishable from the background.
To determine the elemental mapping and elemental/chemical characterization of the QD-PEG NPs, scanning transmission electron microscope in tandem with energy-dispersive x-ray spectroscopy (STEM-EDX) were performed, which simultaneously allows direct correlation of image and quantitative data. However, elemental mapping of the nanoparticles was not successful due to its extremely small dimensions and partly due to its drifting during the characterization process. EDX analysis have shown that QD-PEG NPs comprised of 93.73% cadmium and 6.27% selenide, thus concurring with the manufacturer’s data regarding the core structure and elemental abundance of the QDs (Figure 3.1F).

Morphology of QD-HC NPs were also determined using transmission electron microscopy (TEM) and in comparison to QD-PEG NPs, the former is less structurally defined and lacking sharp edges compared to the latter due to formation of hard protein corona shells; apart from that, there were no major differences in the morphology between the nanoparticles (Figure 3.1C). Average side length of 11 ± 2 nm characterizes most of the QD-HC NPs and the deficit between the sizes of the two nanoparticles was essentially the thickness of the hard protein corona shells.
Figure 3.1: (A) TEM image and (B) size distribution of QD-PEG NPs. (C) TEM image and (D) size distribution of QD-HC NPs. Bar = 50nm. (E) HRTEM image of QD-PEG NPs encircled in red. Scale bar = 2nm. All the NPs were fixed in 2.5% glutaraldehyde solution. (F) EDX spectrum of QD-PEG NPs.
3.3.2 Size separation, estimation and identification of protein corona

QD-PEG NPs were incubated in 55% human plasma to simulate the mean plasma concentration in human blood and the resulting QD-PEG/protein complex that was formed after a series of washing steps to remove weakly adsorbed proteins was termed QD-HC NPs. The mass of hard protein corona adsorbed on QD-HC NPs at concentrations of 15, 30, and 45nM were quantified by performing µBCA assay (Nejadnik and Jiskoot, 2015). By plotting the graph of incrementing nanoparticle concentration against the total bound protein, it was established that protein mass increases at a commensurate level to the increase in NPs’ concentration (Figure 3.2B). This suggests that the quantitative relationship between protein adsorption and NPs depend on the latter’s magnitude of surface area to volume ratio. Incidentally, the proportional increase of proteins relative to NPs indicate that protein adsorption did not reach the saturation level and concentration of human plasma was not a limiting factor in the formation of hard protein corona.

Hard protein corona formed on QD-HC NPs were analyzed using proteomic techniques such as SDS-PAGE, MALDI-TOF/TOF, and LC-MS/MS. Hard protein corona on QD-HC NPs that was subsequently incubated in cell culture medium supplemented with 15% fetal bovine serum was also subjected to the proteomic analysis. This additional analysis was performed to gauge the robustness of hard protein corona since many studies have reported on the evolution of protein corona due to constant association and disassociation of proteins/peptides in serum/plasma containing culture medium (Barrán-Berdón et al., 2013; Ge et al., 2015). More importantly, in in vitro experiments that require
FBS supplemented cell culture medium, additional modifications on the hard corona shells can alter its interaction with the cells, which in turn may affect its cytotoxic and uptake characteristics.

Hard protein coronas formed on QD-HC NPs and QD-HC NPs that was subsequently incubated in cell culture medium supplemented with 15% fetal bovine serum were analyzed using 15% SDS-PAGE gels with coomassie blue (Figure 3.3A) and silver staining (Figure 3.3B) (Hajipour et al., 2014). As predicted, silver staining was more sensitive to the detection of lower volume protein/peptides compared to coomassie blue staining, especially for the lower molecular weight proteins/peptides that were extracted from QD-HC NPs (Figure 3.3C).

L1 on the gel image is QD-HC incubated in human plasma only and L2 is QD-HC incubated in human plasma followed by cell culture medium supplemented with 15% fetal bovine serum. Diluted human plasma (L3) was used as positive control for the gels and reference control for hard protein corona. No bands were observed for the negative controls on (L4) and (L5), indicating that the centrifugation and washing steps were carried out methodically without introducing contaminating protein residues. Based on the qualitative analysis of 15% SDS-PAGE gel image, no glaring differences between the two samples were observed, indicating that the FBS proteins did not alter the hard corona composition of human plasma.
MALDI-TOF/TOF analysis were performed on the four most prominent bands excised from coomassie stained gel (Figure 3.4). The results of MALDI-TOF/TOF analysis concurs with the molecular weight of the predicted proteins in the gel and the four identified proteins based on the increasing order of molecular weights were, Apolipoprotein A-I < Serum Albumin < Complement C3 < Apolipoprotein B-100.

The data obtained from the LC-MS/MS analysis showed similar hard protein corona composition obtained from SDS-PAGE. Thus, the LC-MS/MS results were in agreement with the qualitative analysis of 15% SDS-PAGE gel image in validating the negligible effects of FBS proteins on the hard corona composition of QD-HC nanoparticles. Proteins/peptides identified in MALDI-TOF/TOF analysis matches with the highest scoring proteins/peptides revealed by LC-MS/MS data (Table 3.1). The model of hard protein corona layer present on QD-HC nanoparticles based on the four most abundant proteins identified by mass spectrometry is illustrated in Figure 3.5.
Figure 3.2: (A) BSA standard curve and (B) mass of adsorbed proteins at increasing concentration of nanoparticles.
Figure 3.3: SDS-PAGE analysis of hard protein corona formed on QD-HC nanoparticles: (A) coomassie blue staining, (B) silver staining and (C) comparison between the staining methods in identifying low abundance proteins/peptides. Silver staining was more sensitive to the detection of lower volume protein/peptides compared to coomassie blue staining, especially for the lower molecular weight proteins/peptides as highlighted by the red boxes. M1: molecular weight markers (10-250kD); L1: hard corona (QD-HC); L2: hard corona (QD-HC + DMEM/15% FBS); L3: +ve control (diluted human plasma 1:50); L4: -ve control for washing steps (human plasma only, no NPs); L5: -ve control for washing steps (NPs only, no human plasma); M2: molecular weight markers (1.7-40kD).
Figure 3.4: MALDI-TOF/TOF analysis of the four most abundant hard protein corona (A-D) formed on QD-HC nanoparticles. On the left is a description of the identified proteins from the gel detailing the ascension number, protein name and molecular weight/mass (Da) based on mascot search results from uniprot database.
Table 3.1: LC-MS/MS results of ten most abundant hard protein corona formed on QD-HC NPs.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Score (%)</th>
<th>Molecular Weight</th>
<th>Protein Description</th>
</tr>
</thead>
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<tr>
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</tr>
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</tr>
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<td>K1C9_HUMAN</td>
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Figure 3.5: Hypothetical schematic illustration of QD-PEG nanoparticle transformation in 55% human plasma solution. After washing and centrifugation steps were performed to remove the weakly adsorbed proteins, tightly bound hard protein corona on the nanoparticles were mainly composed of apolipoprotein B-100, complement C3, serum albumin and apolipoprotein A-1 proteins.
3.3.3 Nanoparticle size distribution and agglomeration analysis

The Z-average hydrodynamic diameter and polydispersity index (PdI) of the QDs were measured by dynamic light scattering (DLS) techniques to determine the nanoparticle size distributions; whilst, zeta potential measurements were performed to determine the surface charge of the QDs. The results in the present study have indicated that the hydrodynamic diameter of QDs were larger compared to its primary diameter as determined by TEM analysis. The discrepancy in size measurements were reflective on the method of analysis since the hydrodynamic parameter is usually affected by the surface charge of nanoparticles in colloidal form; whilst, TEM measurement of the nanoparticles were performed in dry conditions. DLS measurements of QD-PEG and QD-HC nanoparticles at various concentrations have revealed that hydrodynamic radiiuses of both nanoparticles were concentration dependent and a positive correlation can be established between these two parameters (Figure 3.6A). Below the 10 nM concentration threshold, the sizes of QDs were not effectively measured using DLS.

Hydrodynamic diameter of QD-HC was consistently larger than QD-PEG at equivalent concentrations and the size inflation in the former is symptomatic of hard protein corona adsorption. QD-HC was composed of heterogeneous protein species with different molecular weight and sizes based on SDS-PAGE, MALDI-TOF/TOF and LC-MS/MS analysis. The current model of hard protein corona formation on QD-HC was categorized into two phases. The primary phase was depicted by smaller proteins/peptides penetrating the crevices of long PEG chains which surrounds the nanoparticle and
adsorbing onto the pockets of vacant surface spaces (Michel et al., 2005). The secondary phase was depicted by larger protein molecules stacking on top of the smaller ones whilst accumulating in between the void of PEG polymers and attaching to its ether repeats (Latza et al., 2017). As a consequence, the PEG chains were prevented from coiling and compressing onto itself, resulting in the thickening of QD-HC as evidenced by the shift towards larger nanoparticle size distribution in DLS data.

The zeta potential values of QD-HC (-10.0 mV) were significantly lower compared to QD-PEG (-2.0 mV) in DPBS for all the concentrations evaluated (Figure 3.6C), although the overall PdI values for the QDs were less than (0.13), indicating monodisperse nanoparticles with a narrow size distribution (Figure 3.6B). In terms of colloidal stability, the current data suggests that PdI gives a better indication of the colloids local entropy state compared to zeta potential values. It has been generally reported in literature that higher absolute zeta potential values indicate increased colloidal stability (Bhattacharjee, 2016). Based on this criteria, QD-PEG having zeta potential values of (0.99 to 1.1 mV) can be classified as neutral nanoparticles and considered highly unstable in physiological solutions; whilst, QD-HC with zeta potential values of (-9 to -10.5 mV) can be classified as anionic nanoparticles and considered relatively stable in physiological solutions. However, the zeta potential results did not correlate decisively with the size distribution curves and PdI values obtained for both QDs with regards to its stability, which shows narrow monomodal distribution curve with low PdI value indicating excellent colloidal stability at physiological pH.
The error in data interpretation to describe the colloidal stability of the QDs can be attributed to the measurement principles involved and the physicochemical characteristics of the individual nanoparticles. Zeta potential measurement does not account for van der Waals attractive forces and steric interactions between nanoparticles that may exist in a colloidal system (Bhattacharjee, 2016). Therefore, low zeta potential values for QD-PEG does not indicate reduced colloidal stability because the steric interaction conferred by high density PEG chains (PEG-5000) on these nanoparticle stabilizes the colloids by increasing its dispersibility. The decrease in zeta potential values for QD-HC was ascribed to hard protein corona formation and many studies have reported on the impartment of negative surface charge to nanoparticles by means of protein adsorption (Monopoli et al., 2011b; Tenzer et al., 2013).

The increased surface charge on QD-HC provides electrostatic repulsion and prevents agglomeration of nanoparticles. It was hypothesized that, the cumulative effect of steric interactions conferred by PEG polymers combined with electrostatic repulsion conferred by protein hard corona would provide QD-HC better dispersion in physiological solution and lowers the entropy of the colloidal system compared to QD-PEG. The data have shown that PdI values of QD-PEG were slightly higher with a broader monomodal distribution curves compared to QD-HC at similar nanoparticle concentrations in DPBS, thus supporting the current hypothesis.
Figure 3.6: Colloidal stability of QD-PEG and QD-HC nanoparticles at various concentrations in DPBS solution. (A) intensity based DLS measurement, (B) PdI measurement and (C) zeta potential measurement. Values are presented as mean ± SD and n=3 for zeta potential measurements.
The stability of QDs were assessed in serum free cell culture medium at various time intervals. In progression of time, the stability of 30nM QD-PEG was affected due to the high ionic strength of the culture medium (Zook et al., 2011). By 15 minutes of incubation, bimodal particle distribution curves were observed indicating the loss of monodispersibility and increased agglomeration of nanoparticles (Figure 3.7A). The PdI increases at each time interval and within 60 minutes of incubation period, the peaks of particle distribution curve had completely shifted to the larger side (Figure 3.7B). Since, steric repulsion from the long PEG brushes provides stabilization in the QD-PEG colloidal system; depolymerization of the PEG chains in high ionic concentration solutions could have reduced the inter-particle repulsion and caused the nanoparticles to agglomerate. On the contrary, 30nM QD-HC was more resistant to changes in the serum free culture medium with the progression of incubation time. Even though the peaks of particle distribution curve had shifted slightly towards the larger side and there was a minor broadening of the curves at increasing time intervals, it retains the monomodal distribution curve configuration; thus indicating a high degree of colloidal stability compared to QD-PEG (Figure 3.8A). Furthermore, after 60 minutes of incubation time, the hydrodynamic diameter of 30nM QD-PEG was larger compared to 30nM QD-HC due to nanoparticle agglomeration in the serum free culture medium. There was also a minor reduction in the zeta potential values of 30nM QD-HC in serum free media compared to DPBS (Figure 3.8C). The differences in zeta potential values was ascribed to the compression of electrical double layer surrounding the nanoparticles in the serum free medium (Roberts et al., 2014). Perturbation to the electrical double layer in the colloid occurred as a result of increased ionic strength, especially with the abundance of divalent ions present in the serum free medium compared to DPBS that was devoid of salt cations such as calcium.
(Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)). Since the prevailing force that provides stability in QD-HC colloidal system was the protein-protein electrostatic repulsion, its decline as a consequence of electrical double layer compression may explain the slight increase in particle size distribution curve.

At higher nanoparticle concentration of 45nM QD-HC, the particle distribution curve had shifted entirely to the larger side without breaching its symmetrical pattern and resembled 30nM QD-PEG’s peak curve position at 60 minutes mark. As the data had indicated earlier, higher nanoparticle concentration tends to increase the particle size distribution due to modulation of intermolecular forces between the nanoparticles. In QD-HC, protein-protein electrostatic repulsion from electrical double layer that surrounds the nanoparticles prevented its agglomeration, however as nanoparticle concentration increases, the thickness of electrical double layer decreases (Tantra et al., 2010). Consequently, the dominance of protein-protein electrostatic repulsion diminishes and van der Waals attraction between the nanoparticles increases, hence raising the frequency of nanoparticle agglomeration. This was evidenced by the reduction in the mean zeta potential value of 45nM QD-HC relative to 30nM QD-HC, thus explaining the decreased colloidal stability of the former in serum free medium. However, the colloidal stability of 45nM QD-HC was still retained as time progresses, albeit minor shifting of the particle distribution peak to the large side similar to 30nM QD-HC. The time progression study has highlighted the stabilizing effect that hard protein corona had imbued to QD-HC in high ionic strength cell culture medium.
Figure 3.7: Colloidal stability of 30 nM QD-PEG nanoparticles at various incubation period in serum free cell culture medium. (A) Intensity based DLS measurement, (B) PdI measurement and (C) zeta potential measurement. Values are presented as mean ± SD and n=3 for zeta potential measurements.
Figure 3.8: Colloidal stability of 30 nM and 45 nM QD-HC nanoparticles at various incubation period in serum free cell culture medium. (A) Intensity based DLS measurement, (B) PdI measurement and (C) zeta potential measurement. Values are presented as mean ± SD and n=3 for zeta potential measurements.
The stability of QD-PEG and QD-HC were assessed in pre-conditioned mediums that were obtained from young and senescent cell cultures to yield accurate model of colloidal behavior in cytotoxicity assay conditions. The dynamic exchange of biologics and organic molecules between proliferating cells and extracellular bulk medium occurs as result of nutrient consumption and metabolic waste excretion. In proliferating cells, the pH of extracellular medium tends to fluctuate due to increased exchange in nutrients, organic acids, and proteins (Palm and Thompson, 2017). Therefore, the preconditioned medium from young cells could vary marginally relative to fresh serum free medium, which may affect the colloidal stability of the nanoparticles. The data has shown that the stability of QD-PEG was only affected in pre-conditioned medium from young cells at higher confluency (50%) in both fibroblast and epithelial cells (Figure 3.9A). The QD-PEG were polydispersed as evidenced by the increment in PdI value and particle distribution curve stretching to the larger side; meaning the nanoparticles were slightly more agglomerated in the pre-conditioned medium from young cells at higher confluency (50%) compared to fresh serum free medium (Figure 3.9B).

There was also a decrease in the zeta potential values of QD-PEG (-8.0 mV) in these pre-conditioned mediums compared to the other setups (Figure 3.9C). Based on the results, it was hypothesized that the agglomeration of QD-PEG in these pre-conditioned mediums was due to heteroaggregation between the nanoparticles and extracellular proteins in tandem with the existing homoaggregation between the nanoparticles (Liu et al., 2013). Unlike the controlled preparation of hard protein corona using plasma proteins in the current experiment, whereby smaller protein molecules fill up the void of
nanoparticle’s exposed surface area and crevices of PEG oligomers; the extracellular proteins in the pre-conditioned medium were probably larger and the dynamics of interaction would have been different. Instead of fully adsorbing to individual nanoparticles and providing uniform electrostatic repulsion (Figure 3.11B), many smaller nanoparticles would have adsorbed to the larger proteins amounting to increased agglomeration instead of dispersion (Figure 3.11C). Extracellular protein adsorption would also explain the observed increase in negative zeta potential value of QD-PEG. Furthermore, QD-PEG in pre-conditioned medium from young cells at higher confluency (50%) had higher zeta potential values relative to QD-HC in all the mediums, indicating limited exposure of protein molecules within the nanoparticle-protein complex and reinforcing the earlier stated hypothesis of heteroaggregation between nanoparticles and extracellular proteins.

The pre-conditioned mediums from lower confluency of proliferating cells (10%) did not affect the stability of QD-PEG significantly. Higher confluency of proliferating cells (50%) tend to modify the bulk structure of extracellular medium at a faster rate compared to lower confluency of proliferating cells (10%), hence the less interference to the colloidal stability of QD-PEG in the latter. The pre-conditioned medium from senescent and contact-inhibited young cells did not affect the stability of QD-PEG significantly. This could be due to the fact that these cells were not proliferating even though they were metabolically active (Marthandan et al., 2014). Due to non-proliferation, expansion of energy was reduced, hence less metabolic waste would have been released.
within the same time span compared to young cells. Therefore, the composition of the extracellular medium was not altered considerably relative to fresh serum free medium.

In the case of 30 & 45 nM QD-HC, the nanoparticles were very stable in all the pre-conditioned mediums and no significant changes were observed compared to fresh serum free medium (Figure 3.10). Even in the pre-conditioned mediums from higher confluency of proliferating cells (50%) that were presumed to contain a considerable amount of extracellular protein residues, the stability of QD-HC was not significantly affected. It was conjectured that electrostatic repulsion between the extracellular proteins and hard protein corona layer on QD-HC would have prevented the agglomeration of nanoparticles. After considering all the experimental parameters and conditions that could have affected the nanoparticle stability in the current cytotoxicity study, it was apparent that QD-HC nanoparticles were more stable than QD-PEG in colloidal form.
Figure 3.9: Colloidal stability of 30 nM QD-PEG nanoparticles at 60 min incubation period in serum free and preconditioned cell culture medium. (A) Intensity based DLS measurement, (B) PdI measurement and (C) zeta potential measurement. Values are presented as mean ± SD and n=3 for zeta potential measurements.
Figure 3.10: Colloidal stability of 30 nM and 45 nM QD-HC nanoparticles at 60 min incubation period in serum free and preconditioned cell culture medium. (A) Intensity based DLS measurement, (B) Pdl measurement and (C) zeta potential measurement. Values are presented as mean ± SD and n=3 for zeta potential measurements.
**Figure 3.11:** Hypothetical schematic illustration of nanoparticle’s aggregation models. (A) homoaggregation among QD-PEG NPs, (B) homoaggregation among QD-HC NPs and (C) heteroaggregation between QD-PEG NPs and extracellular proteins.
3.3.4 Nanoparticle gravitational sedimentation analysis

Gravitational sedimentation analysis was performed on 30nM QD-PEG, 30nM QD-HC and 45nM QD-HC nanoparticles in serum free medium to better understand cell uptake kinetics. These three difference concentrations of NPs were selected based on their cytotoxic potential against the cells (section 6.3.1). In the current cell uptake experimental settings, monolayer of cells were located at the bottom of the culture wells, which is the interface point between the cells and nanoparticles. Therefore, gravitational sedimentation of nanoparticles takes precedence over diffusion kinetics in the cell culture system and warranting the analysis of this phenomenon (Cho et al., 2011).

Mean size of individual nanoparticle sediments that were formed as a result of gravitational force at 30 and 90 minutes of incubation time were measured using AFM (Figure 3.12). The data have shown that the smallest sediment size on average (95 nm) with the highest volume (78.3%) was formed by 30nM QD-HC after 30 minutes of incubation period; whilst the largest sediment size on average (213 nm) with the highest volume (77%) was formed by 30nM QD-PEG and followed very closely by 45nM QD-HC within the same time frame. The sediment sizes of 30nM QD-PEG were 2.4 fold larger compared to 30nM QD-HC; whilst the sediment sizes of 45nM QD-HC were 2 fold larger compared to 30nM QD-HC based on the highest volume of sediments with a threshold above 70%.

The diffusion kinetics and dispersibility of nanoparticles in culture medium influences the sediment formation (Allouni et al., 2009); as such, DLS data should elucidate the sedimentation process of the nanoparticles. Based on the hydrodynamic diameter of nanoparticles, 30nM QD-HC was minuscule in comparison with 30nM QD-
PEG and 45nM QD-HC in serum free media, while the latter nanoparticles have similar sizes. A correlation between hydrodynamic diameter and sediment size was indicated and a straightforward explanation can be made on the basis that larger sized nanoparticles have higher density; thus, accelerating its gravitational settlement to the bottom of culture wells and forming larger sediments compared to smaller nanoparticles at any given time.

Since the hydrodynamic diameters of 30nM QD-PEG and 45nM QD-HC were similar, their corresponding highest volume of sediment sizes were also comparable. The effect of hydrodynamic size of nanoparticles on sediment formation was more apparent with progression of time (Figure 3.13). As the incubation period was increased to 90 minutes, volumetric increase in sedimentation sizes was observed for all the nanoparticles compared to the previous 30 minutes (Figures 3.14 & 3.15). More importantly, the increase in sedimentation size of 30nM QD-PEG (466 nm) were 3.7 fold larger compared to 30nM QD-HC (127 nm); whilst the increase in sedimentation size of 45nM QD-HC (440 nm) were 3.5 fold larger compared to 30nM QD-HC (127 nm) in 90 minutes based on the highest volume of sediments with a threshold above 70%.

The results have indicated that the probability of nanoparticles piling up on existing sediments increases when the incubation time was extended and larger nanoparticles tend to form sediments at a faster rate compared to smaller nanoparticles due to stronger gravitational exertion on the former.
Figure 3.12: Comparison of mean size of individual QD-PEG and QD-HC nanoparticle sediments after (A) 30min and (B) 90 min of incubation in cell culture medium. Right Y axis indicates the volume of sediments corresponding to the mean size of individual sediments and both values are depicted as adjacent graph bars. Data are represented as mean ± SD. n=3 for each group.
Figure 3.13: Microscopic images of QD-PEG NPs (left) and its corresponding AFM scans (right). The images from top to bottom (A-C) depicts increasing incubation time and its effect on the gravitational sedimentation of NPs. A: 30 minutes, B: 60 minutes, C: 90 minutes.
Figure 3.14: AFM images and height profiles of 30nm QD-PEG pertaining to gravitational sedimentation size after 90 minutes of incubation time in cell culture medium.
Figure 3.15: AFM images and height profiles of 30nm QD-PEG pertaining to gravitational sedimentation size after 30 minutes of incubation time in cell culture medium.
3.3.5 Nanoparticle photostability analysis

QD photostability was measured with fluorescence spectrophotometer at cellular cytosolic and lysosomal pH after 4 hours of incubation. Comparison was made between 30nM QD-PEG and 30nM QD-HC NPs based on their cytotoxic potential against the cells (section 6.3.1). At equal concentrations, these two NPs have induced different cytotoxic effect on the cells, which could be attributed to the rate of QD disintegration in cellular milieu. The process of QD disintegration can be directly correlated to the photostability of the QDs.

The data have shown that, at cytosolic pH 7.2, there was no significant difference in the fluorescence intensity between 30nM QD-PEG and 30nM QD-HC, indicating that hard protein corona formation did not alter the photochemical properties of QD-HC (Figure 3.16). In fact, the mean intensity value for QD-HC was marginally higher than QD-PEG, which were attributed to increased agglomeration of the latter in physiological buffer as shown in DLS data. Agglomeration of nanoparticles tend to distort focused fluorescent measurements due to non-uniform colloidal dispersibility in cuvettes. Another possibility is that, hard protein corona layer on QD-HC had prevented ionic attacks on its surface that would have otherwise changed the surface charge states of the QD, resulting in loss of fluorescence (Sahoo et al., 2007).

At lysosomal pH 4.5, the fluorescent intensity of both nanoparticles were reduced significantly (P < 0.001) compared to measurements that were performed at cytosolic pH
7.2. It was hypothesized that in highly acidic and oxidative conditions, degradation of the nanoparticles was the causative factor for the reduced fluorescent intensity. In QD-PEG, the core composition of the nanoparticle that generated the fluorescent signals upon excitation by UV light were the Cd/Se/Te complex; thus, degradation of the core composition will result in diminishing fluorescence quantum yield.

However, the core composition was protected by a thin ZnS semiconductor shell that provides surface passivation and methoxy-polyethylene glycol (PEG-5000) coating that improves colloidal stability of the nanoparticles in biological medium (Dabbousi et al., 1997). Therefore, degradation of the QD’s core must be preceded by desorption of PEG and dissolution of ZnS shell; whilst in QD-HC, hard protein corona layer must be disintegrated prior to PEG desorption and ZnS dissolution.

The loss of fluorescence was also significant (P < 0.001) between 30nM QD-PEG and 30nM QD-HC at lysosomal pH 4.5 with the former having the lower intensity; implying that the degradation of nanoparticles was greater in 30nM QD-PEG. The only difference between these nanoparticles was the presence of protein hard corona layer on QD-HC, suggesting that this assembly have enabled the nanoparticles to withstand highly oxidative or acidic conditions and inadvertently delayed its degradation (Figure 3.17).

To further imitate lysosomal conditions, co-incubation of proteinase K (protease) with QD-HC in artificial lysosomal fluid (ALF) was performed to degrade the hard protein
corona layers on QD-HC. Proteinase K is an enzyme that is commonly used in molecular biology for general digestion of proteins due to its broad specificity. Although in actuality, more than 50 different hydrolytic enzymes have been reported to reside in lysosomes that could catalyze the degradation process at an even faster rate (Schroder et al., 2010). As anticipated, the fluorescent intensity of QD-HC was reduced significantly ($P < 0.001$) compared to its incubation in ALF without the protease; nevertheless, having higher fluorescent intensity than QD-PEG in ALF. In essence, hard protein corona had imbued robustness to QD-HC without altering its photonic characteristics.
**Figure 3.16:** Photostability of QD-PEG and QD-HC at cell cytosolic and lysosomal pH after 4 hours of incubation. Excitation wavelength was 488 nm and emission was measured at 660-735 nm. Data are represented as mean ± SD. n=3 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Figure 3.17: Hypothetical schematic illustration of QD-HC nanoparticle gradual disintegration in ALF/protease solution. A-D, depolymerization of PEG chains, proteolysis of layers of embedded peptides/proteins, dissolution of ZnS coating and finally degradation of core quantum materials Cd/Se/Te.
3.4 Conclusion

The current results have shown that protein corona was able to form on pristine PEGylated quantum dots (QD-PEG) and subsequently transform its physicochemical properties. Based on extensive characterization, it was determined that changes to the physicochemical properties had in turn affected the colloidal stability of QD-PEG in a significant manner.

At proportionate levels of nanoparticle concentration (30nM QD-PEG and 30nM QD-HC), hard protein corona had imbued distinct colloidal properties to the nanoparticle that were better suited for nanomedical applications in terms of: (1) enhanced photostability at extreme pH conditions, (2) greater resistance to changes in extracellular medium that induces agglomeration and gravitational sedimentation, and (3) increased robustness to degradation and leaching of QDs’ core material at extreme pH conditions.

At disproportionate levels of nanoparticle concentration (30nM QD-PEG and 45nM QD-HC), the colloidal characteristics with regards to agglomeration and gravitational sedimentation of both nanoparticles were comparable. This particular parameter of colloid is important in the current study because it embodies the tangible size of nanoparticles in the cell culture medium during cytotoxicity experiments rather than the theoretical size of individual nanoparticles. Therefore, any interference that may exist during cytotoxicity testing attributable to size-dependent toxic effects was kept at a bare minimum.

30nM QD-PEG, 30nM QD-HC and 45nM QD-HC were selected for cytotoxicity experiment to highlight the differences between these nanoparticles.
CHAPTER FOUR: CELL CULTURING, MAINTENANCE AND INDUCTION
OF CELLULAR SENESCENCE

4.1 Introduction

Cellular senescence is the embodiment of organismal ageing and explication of this process is occurring at a rapid pace owing to the sophistication of biomedical tools and improvement in research techniques. Even though, there are many theories explaining the senescence process or its causative factors, the ultimate manifestation of this complex event is typically characterized by altered cellular functions and morphogenesis. In the current study, the impact of nanoparticles on senescent and non-senescent cell population was examined.

To accomplish the aforementioned objective, it was paramount to establish reliable \textit{in vitro} senescent cell models for data collection and analysis. Thus, normal embryonic diploid cells of epithelial and fibroblast origins were induced into entering senescence state by three different methods: (1) Replicative senescence via serial passaging of cells, (2) Oxidative stress induced premature senescence via hydrogen peroxide treatment, and (3) Genotoxic stress induced premature senescence via doxorubicin treatment. The induced cells were then subjected to biochemical characterization to determine if the majority of cell population had achieved deep senescence at or above pre-set threshold levels. Concurrently, senescence biomarker assays were performed on untransformed primary fibroblast cells of different age groups to determine the correlation between replicative/premature senescence (\textit{in vitro}) and chronological lifespan (\textit{ex vivo}) as illustrated in Figure 4.1.
**Figure 4.1:** Flow chart illustrating the cell ageing methods deployed in the current study
4.2 Materials and methods

4.2.1 Cell culture

4.2.1(a) Normal human lung fibroblast (IMR90)

IMR90 fibroblast cells from human fetal lung (CCL-186) were purchased from ATCC (ATCC Cell lines Service, USA). IMR90 cells were used for aging experiments. The cells were obtained at passage number five and cultured in DMEM with 15% (v/v) Fetal Bovine Serum (FBS) and 1% Penicilin-Streptomycin antibiotics.

4.2.1(b) Normal human colon epithelial (CCD841CoN)

CCD841CoN epithelial cells from human fetal colon (CRL-1790) were purchased from ATCC (ATCC Cell lines Service, USA). CCD841CoN cells were used for aging experiments. The cells were obtained at passage number seven and cultured in DMEM with 15% (v/v) Fetal Bovine Serum (FBS) and 1% Penicilin-Streptomycin antibiotics.

4.2.1(c) Untransformed adult human lung fibroblasts (AG02262)

Untransformed Adult human lung fibroblasts (AG02262) were obtained from the National Institute of Ageing, Cell and Culture Repository, Coriell Institute for Medical Research (Camden, NJ). AG02262 cells were obtained at population doubling six and were cultured in DMEM supplemented with 15% FBS and 1% Penicilin-Streptomycin antibiotics. The cultured cells were maintained in an incubator set to an atmosphere of 37°C and 5% CO₂.
4.2.1(d) Untransformed adult human lung fibroblasts (AG02603)

Untransformed adult human lung fibroblasts (AG02603) were obtained from the National Institute of Ageing, Cell and Culture Repository, Coriell Institute for Medical Research (Camden, NJ). AG02603 cells were obtained at population doubling three and were cultured in DMEM supplemented with 15% FBS and 1% Penicillin-Streptomycin antibiotics. The cultured cells were maintained in an incubator set to an atmosphere of 37°C and 5% CO₂.

4.2.1(e) Untransformed fetal human lung fibroblasts (AG04450)

Untransformed fetal human lung fibroblasts (AG04450) were obtained from the National Institute of Ageing, Cell and Culture Repository, Coriell Institute for Medical Research (Camden, NJ). AG04450 cells were obtained at population doubling seven and were cultured in DMEM supplemented with 15% FBS and 1% Penicillin-Streptomycin antibiotics. The cultured cells were maintained in an incubator set to an atmosphere of 37°C and 5% CO₂.

4.2.2 Senescence induction of IMR90 and CCD841CoN cells

4.2.2(a) Replicative senescence

Replicative senescence of CCD8411CoN and IMR90 cells were induced by serial passages performed at 3 day intervals with a seeding density of 3 x 10⁵ cells/ml in T25 flasks (5 ml). The number of population doubling level (PDL) was calculated using the
formula: \( PDL = \log_2 \left( \frac{AY}{AX} \right) \); where \( AX \) represents the number of cells seeded and \( AY \) represents the number of cells harvested. PDL 12 and PDL 48 were considered as young and senescent cells in CCD8411CoN; PDL 14 and PDL 55 were considered as young and senescent cells in IMR90. The cells were quantified using cell senescence biomarker assays.

4.2.2(b) Genotoxic stress induced premature senescence

CCD8411CoN and IMR90 cells were exposed to increasing concentrations of doxorubicin for 3 days and then recultured in fresh medium for 24 h before WST-1 cell viability assay was performed to determine sublethal concentrations of doxorubicin for senescence induction without loss of cell viability. Three different concentrations of 40, 70 and 100nM were used to study the effectiveness of doxorubicin to induce premature senescence on CCD8411CoN and IMR90 cells. Cells were seeded at low density in the culture plate to accommodate for cell flattening and size increase with incubation time. Cells were incubated with complete medium containing 40, 70 and 100nM doxorubicin for 7 days with fresh doxorubicin change every 3 days and were quantified in situ 16 days later using cell senescence biomarker assays.

4.2.2(c) Oxidative stress induced premature senescence

CCD8411CoN and IMR90 cells were exposed to increasing concentrations of \( \text{H}_2\text{O}_2 \) for 2 hours and then recultured in fresh medium for 24 h before WST-1 cell viability assay was performed to determine sublethal concentrations of \( \text{H}_2\text{O}_2 \) for senescence
induction without loss of cell viability. Three different concentrations of 10, 30 and 50µM were used to study the effectiveness of H$_2$O$_2$ to induce premature senescence on CCD8411CoN and IMR90 cells. Cells were seeded at low density in the culture plate to accommodate for cell flattening and size increase with incubation time. Cells were treated with a 2-hour pulse of 10, 30 and 50µM H$_2$O$_2$ twice within 3 days apart of each treatment and were quantified in situ 16 days later using cell senescence biomarker assays.

4.2.3 Cellular senescence biochemical characterization

4.2.3(a) 5-bromo-2’-deoxyuridine (BrdU) incorporation assay

The medium from cells were removed from the wells and the cells were washed twice with PBS. 10 µM BrdU labeling solution in cell culture medium were added per 35-mm dish and the cells were incubated at 37°C for 24 h. After incubation, the labelling solutions were removed and the cells were washed 3X with PBS for 2 minutes each. After washing, 1 mL of 3.7% formaldehyde in PBS were added to each well and incubated for 15 minutes at room temperature to fix the cells. After incubation, the fixing solutions were removed and the cells were washed 3X with PBS for 2 minutes each. After washing, 1 mL of Triton X-100 permeabilization buffer were added to each well and incubated for 20 minutes at room temperature.

After incubation, the permeabilization buffer were removed and 1 mL of 2M HCl were added to each well and incubated for 30 minutes at room temperature. After incubation period, 1 mL phosphate/citric acid buffer, pH 7.4 were added to each well and
incubated for another 10 minutes at room temperature. After incubation, the solutions were removed and the cells were washed 3X with of Triton X-100 permeabilization buffer for 2 minutes each. After washing, 1 mL blocking solution (1% BSA, 0.1% triton X-100, 0.3M Glycine in PBS) were added to each well and incubated for 30 minutes at room temperature. After incubation, blocking solutions were removed and antibody solution were added and incubated in the dark for 2h at room temperature. After incubation period, the solutions were removed and washed for three times for 5 minutes with PBS.

Finally, Fresh PBS were added in all the wells and observed under fluorescence microscope at 200X magnification. Anti-BrdU monoclonal antibodies (Alexa fluor 647) were used to identify proliferating IMR90, AG04450, AG02603 and AG02262 cells at excitation/emission wavelength of 633/670 nm. Anti-BrdU monoclonal antibodies (Alexa fluor 488) were used to identify proliferating CCD8411CoN cells at excitation/emission wavelength of 488/520 nm. 100 cells were scored per treatment from 6 replicates of 3 independent experiments.

4.2.3(b) Senescence associated β-galactosidase (SA-β-Gal) assay

Sub-confluent cultures with comparable cell density were used in β-galactosidase assay to avoid false positive staining. The medium was removed from the wells and the cells were washed twice with PBS. After washing, 1 mL of 3.7% formaldehyde in PBS were added to each well and incubated for 3-5 minutes at room temperature to fix the cells. After incubation, the fixing solutions were removed and the cells were washed twice
with PBS. 1–2 mL of X-gal staining solution (1 mg/ml X-gal, 40 mM sodium citrate buffer (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂) were added per 35-mm dish and incubated at 37°C for 12–16 h in a non CO₂ incubator.

After the incubation time, the staining solution were removed and cells were washed twice with PBS. Fresh PBS were added in all the wells and observed under bright field/ phase contrast microscope at 200X magnification. 100 cells were scored per treatment from 6 replicates of 3 independent experiments.

4.2.3(c) Senescence associated heterochromatin foci (SAHF) assay

The medium from cells were removed from the wells and the cells were washed twice with PBS. 1–2 mL of Hoechst 33342 (1 µg/ml) staining solution in PBS were added per 35-mm well. The cells were incubated in staining solution for 20 minutes protected from light. After the incubation time, the staining solution were removed and cells were washed twice with PBS. Fresh PBS were added in all the wells and observed under fluorescence microscope at excitation/emission wavelength of 350/461 nm (400X magnification). 100 cells were scored per treatment from 6 replicates of 3 independent experiments.
4.2.4 Statistical analyses

Statistical analysis was performed based on one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer post hoc test of significance by using Prism® 5.0 software (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered to be statistically significant. Cytotoxicity results are expressed as mean ± SD of one representative experiment performed in 6 replicates, and the experiments were repeated three times. GraphPad Prism® 5.0 software (GraphPad, San Diego, CA, USA) was used to calculate the concentrations associated with 50% viability (IC50 values) with 95% confidence limits using a Hill function nonlinear regression analysis.
4.3 Results and Discussion

4.3.1 Population doubling level to reach deep senescence

4.3.1(a) Cell growth profile

Induction of replicative senescence on IMR90 and CCD841CoN cells were performed via serial passaging of cultured cells. Scaling of cells in cultures were calculated by measuring its population doublings and hayflick limit of the cells were determined by plotting graph of population doublings against time. Hayflick limit is the nexus point upon which the cells’ population doublings came to a halt as a consequence of serial passaging (Shay and Wright, 2000). The region of growth curve on the graph that is parallel to horizontal axis indicates cessation of detectable cell population growth and corresponds to the cells’ hayflick limit.

IMR90 cells reached hayflick limit at population doubling 55 after spanning over 170 days in culture (Figure 4.2). Post 200 days in culture, IMR90 cells were in the region of deep senescence, wherein population doublings tethered at the same level for 30 days straight without significant increase. Cells in the deep senescent state unveiled different morphological features compared to proliferating cells such as size expansion and formation of stress granules (Figure 4.2 B, C). Proliferating cells at population doublings 1 to 30 that lies on the linear ascension of the growth curve were considered as young cells, wherein cells in the deep senescence region of growth curve were considered as senescent cells in this experiment.
CCD841CoN cells reached hayflick limit at population doubling 48 after spanning over 160 days in culture (Figure 4.3). Post 190 days in culture, CCD841CoN cells were in the region of deep senescence, wherein population doublings tethered at the same level for 30 days straight without significant increase. Cells in the deep senescent state unveiled different morphological features compared to proliferating cells such as size expansion (Figure 4.3 B, C). Proliferating cells at population doublings 1 to 27 that lies on the linear ascension of the growth curve were considered as young cells, wherein cells in deep senescence region of growth curve were considered as senescent cells in this experiment.
Figure 4.2: (A) Growth curve of IMR90 cells. Morphology of cells at (B) rapid proliferation phase (C) deep senescence phase. Scale bar = 50µm.
Figure 4.3: (A) Growth curve of CCD841CoN cells. Morphology of cells at (B) rapid proliferation phase (C) deep senescence phase. Scale bar = 50µm.
4.3.1(b) BrdU incorporation assay analysis

BrdU based cell proliferation assay was performed on serially passaged cells at different population doubling levels (Figure 4.4). The percentage of cells that scored positive for BrdU incorporation was denoted by each column on the graph. Quintessentially, each column signifies the amount of cycling cells within its own population. Suppression of cell proliferation indicates that the viable cells have entered cell cycle arrest, which is a hallmark of cellular senescence. In this experiment, proliferating cells falling below the threshold level of 10% were considered successful for the induction of premature senescence. The difference between PDL 12 and PDL 48 of CCD841CoN cells was highly significant (P < 0.001). Following repeated passages of culture, there was a reduction of 77.67% in cycling cells at PDL 48 compared to PDL 12. At PDL 48 the percentage of proliferating cells were less than 8%, thus approximately 92% of the viable cell population were in cell cycle arrest. This was in stark contrast to cells at PDL 12, whereby 88% of the cells in its population were cycling. Hence, serial passaging had induced most of the cells in CCD841CoN populous to undergo replicative senescence at PDL 48 (Figure 4.4A).

The difference between PDL 14 and PDL 55 of IMR90 cells was highly significant (P < 0.001). Following repeated passages in culture, there was a reduction of 82% in cycling cells at PDL 55 compared to PDL 14. At PDL 55 the percentage of proliferating cells were less than 9%, thus approximately 91% of the viable cell population were in cell cycle arrest. This was in stark contrast to cells at PDL 14, whereby 90.66% of the cells in its population were cycling. Hence, serial passaging had induced most of the cells in IMR90 populous to undergo replicative senescence at PDL 55 (Figure 4.4B).
Figure 4.4: Quantification of proliferating cells in (A) CCD841CoN and (B) IMR90 cultures at different population doubling levels. 5-Bromo-2’-deoxyuridine (BrdU) based cell proliferation in the cultures were detected by immunofluorescence. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.3.1(c) SA-β-galactosidase assay analysis

SA-β-galactosidase assay was performed on serially passaged cells at different population doubling levels (Figure 4.5). The percentage of cells that scored positive for SA-β-galactosidase staining was denoted by each column on the graph. Quintessentially, each column signifies the amount of senescent cells within its own population. In this experiment, threshold level exceeding 80% of SA-β-galactosidase expression within the cell population were considered successful for the induction of premature senescence.

The difference between PDL 12 and PDL 48 of CCD841CoN cells was highly significant (P < 0.001). Following repeated passages in culture, there was an 80% increase in the expression of SA-β-galactosidase at PDL 48 compared to PDL 12. Thus, at PDL 48, 92% of the cells within its population were senescent compared to the lower quantity of senescent cells spotted at PDL 12, which was less than 12%. Hence, serial passaging has induced most of the cells in CCD841CoN populous to undergo replicative senescence at PDL 48 (Figure 4.5A).

The difference between PDL 14 and PDL 55 of IMR90 cells was highly significant (P < 0.001). Following repeated passages in culture, there was an 83% increase in the expression of SA-β-galactosidase at PDL 55 compared to PDL 14. Thus, at PDL 55, 92.33% of the cells within its population were senescent compared to the lower quantity of senescent cells spotted at PDL 14, which was less than 10%. Hence, serial passaging has induced most of the cells in IMR90 populous to undergo replicative senescence at PDL 55 (Figure 4.5B).
Figure 4.5: Quantification of SA-β-galactosidase expression in (A) CCD841CoN and (B) IMR90 cells at different population doubling levels. SA-β-galactosidase activity in the cultures were cytochemically detected at pH 6. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.3.1(d) SAHF assay analysis

SAHF assay was performed on serially passaged cells at different population doubling levels (Figure 4.6). This test measures the amount of senescent cells present in the cell population based on the formation of SAHF that is detectable using nuclear stain, Hoechst. As the cells are induced to undergo senescence, SAHF formation will increase within the cell population. The percentage of cells that scored positive for SAHF formation was denoted by each column on the graph. Quintessentially, each column signifies the amount of senescent cells within its own population. In this experiment, threshold level exceeding 80% of SAHF formation within the cell population were considered successful for the induction of premature senescence.

The difference between PDL 12 and PDL 48 of CCD841CoN cells was highly significant (P < 0.001). Following repeated passage of the cultures, there was a 79.67% increase in the expression of SAHF at PDL 48 compared to PDL 12. Thus, at PDL 48, 83.66% of the cells within its population were senescent compared to the lower quantity of senescent cells spotted at PDL 12, which was less than 4%. Hence, serial passaging has induced most of the cells in CCD841CoN populous to undergo replicative senescence at PDL 48 (Figure 4.6A).

The difference between PDL 14 and PDL 55 of IMR90 cells was highly significant (P < 0.001). Following repeated passage of the cultures, there was a 78% increase in the expression of SAHF at PDL 55 compared to PDL 14. Thus, at PDL 55, 81% of the cells within its population were senescent compared to the lower quantity of senescent cells spotted at PDL 14, which was less than 3%. Hence, serial passaging has induced most of the cells in IMR90 populous to undergo replicative senescence at PDL 55 (Figure 4.6B).
Figure 4.6: Quantification of SAHF formation in (A) CCD841CoN and (B) IMR90 cells at different population doubling levels. SAHF formation in cultures were detected by Hoechst 33342 nuclear staining. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.3.2 Effective concentration of doxorubicin and hydrogen peroxide for cell ageing

Replicative senescence is the most common method employed to induce in vitro cellular senescence for the study of mammalian cell ageing (Campisi, 1997). Replicative senescence requires repeated serial passaging of cells until the cells undergo senescence, which may take many months to achieve. Besides replicative senescence, other methods that induce premature senescence on cells by way of oxidative stress and genotoxic stress can be utilized to generate the senescent phenotype (Zhang et al., 2011b; Dimozi et al., 2015). These methods accelerate the ageing process and induce senescence much more rapidly than replicative senescence. The reason being that, conditions that influence cellular senescence in typical in vitro culture is amplified and exaggerated when the cells are cultured at elevated stress levels promoted by the senescence inducing agents. Another reason could be that the mammalian cells may engage alternate molecular pathways or cellular process than it does with replicative senescence to enter the state of senescence.

In this experiment, oxidative stress via H₂O₂ and genotoxic stress via doxorubicin were used to induce premature senescence on CCD8411CoN and IMR90 cells. First, cytotoxicity assay was performed on both cells to determine the sublethal concentration of H₂O₂ and doxorubicin (Figure 4.7). Then, the chosen concentrations of these chemicals were used to age the cells for a predetermined time. Finally, senescence assays were performed on the treated cells to determine the combination of concentrations and/or methods that yields the best results in senescence induction.
The cytotoxicity results for H$_2$O$_2$ have shown that concentrations below 50µM did not affect the viability of the CCD8411CoN and IMR90 cells (Figure 4.7A). Beyond 50µM the viability of both cells decreased in a concentration dependent manner. CCD8411CoN cells that were exposed to H$_2$O$_2$ within the concentration range of 70-200µM underwent apoptosis and detached within a few hours of exposure. IMR90 cells that were exposed to H$_2$O$_2$ within the concentration range of 60-200µM underwent apoptosis and detached within a few hours of exposure. Exposure of both cells to H$_2$O$_2$ concentrations of 200µM and beyond resulted in necrosis. Since the sublethal concentrations of H$_2$O$_2$ tethers at 50µM or under and for the sake of simplicity, three different concentrations of 10, 30 and 50µM were chosen to study the effectiveness of H$_2$O$_2$ to induce premature senescence on both cells.

The cytotoxicity results for doxorubicin have shown that concentrations below 100 nM and 110 nM did not affect the viability of the CCD8411CoN and IMR90 cells, respectively (Figure 4.7B). CCD8411CoN cells that were exposed to doxorubicin within the concentration range of 150-250 nM underwent apoptosis and detached within 3 days of exposure. IMR90 cells that were exposed to doxorubicin within the concentration range of 180-250 nM underwent apoptosis and detached within 2.5 days of exposure. Exposure of both cells to doxorubicin concentrations of 300 nM and beyond resulted in necrosis. Since the sublethal concentrations of doxorubicin tethers at 100nM or under and for the sake of simplicity, three different concentrations of 40, 70 and 100nM were chosen to study the effectiveness of doxorubicin to induce premature senescence on both cells.
Figure 4.7: Toxicity graph of (A) hydrogen peroxide and (B) doxorubicin treatment on young IMR90 and CCD841CoN cells. Toxicity measurement was performed using WST-1 colorimetric assay with different concentrations of hydrogen peroxide and doxorubicin at various incubation times. The highest concentration of hydrogen peroxide and doxorubicin tested for each experiment was 400µM and 500nM, respectively. Data are represented as mean ± SD. n=6 for each group.
4.3.2(a) Cell growth profile

Cell growth curves of doxorubicin and H$_2$O$_2$ treated cells were plotted by quantifying the total cell count against time. The cell count of untreated control represents the total number of cells that will yield under normal circumstances. Based on the graphs, the growth rate of H$_2$O$_2$ treated IMR90 and CCD841CoN cells were significantly retarded (P < 0.001) in a dose dependent manner in the order of 50µM >30µM >10µM compared to untreated control. In a similar pattern, the growth rate of doxorubicin treated IMR90 and CCD841CoN cells were significantly retarded (P < 0.001) in a dose dependent manner in the order of 100nM >70nM >40nM compared to untreated control. The reduction in cell number due to the toxic effects of chemicals can be ruled out as the cells remained viable at these doses based on the previous cytotoxicity analysis.

In IMR90 cells, the divergence of 50µM H$_2$O$_2$ growth curve relative to the other doses transpired after day 10 of treatment, while the divergence between 30µM and 10µM H$_2$O$_2$ curves transpired after day 20 of treatment (Figure 4.8A). The divergence amongst the growth curves indicate significant drop (P < 0.001) in cell population relative to the ascending curve(s). From day 21 and beyond, the 50µM H$_2$O$_2$ growth curve on the graph was parallel to horizontal axis, indicating cessation of detectable cell population growth; whilst the inclining curves of the other doses have indicated still growing population of IMR90 cells albeit at a much reduced rate compared to the earlier days.
Accompanying the altered cell growth pattern was the overt changes to the morphological features of IMR90 cells such as size expansion and formation of stress granules (Figures 4.8B-D). The transformation was apparent on large majority (<92%) of cells treated with 50µM H₂O₂ after 25 days of exposure while the other doses exhibited commensurate levels of transformation at much latter stages; 30 and 45 days of exposure to 30µM and 10µM of H₂O₂ respectively.
Figure 4.8: (A) Growth curve of IMR90 cells after exposure to hydrogen peroxide treatments at different concentrations. Morphology of cells at (B) 10µM, (C) 30µM, and (D) 50µM hydrogen peroxide treatments. Accompanying the altered cell growth pattern was the overt changes to the morphological features of IMR90 cells such as size expansion and formation of stress granules. Magnification, 200X. Data are represented as mean ± SD. n=3 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
In CCD841CoN cells, the divergence of 50µM H₂O₂ growth curve relative to the other doses transpired after day 10 of treatment, while the divergence between 30µM and 10µM H₂O₂ curves transpired after day 20 of treatment (Figure 4.9A). The divergence amongst the growth curves indicate significant drop (P < 0.001) in cell population relative to the ascending curve(s). From day 21 and beyond, the 50µM H₂O₂ growth curve on the graph was parallel to horizontal axis, indicating cessation of detectable cell population growth; whilst the inclining curves of the other doses have indicated still growing population of CCD841CoN cells albeit at a much reduced rate compared to the earlier days.

Alteration to the cell growth pattern was accompanied by enlargement of CCD841CoN cells similar to IMR90 cells but formation of stress granules was not apparent in the former (Figures 4.9B-D). The size increase was observed on large majority (< 95%) of cells treated with 50µM H₂O₂ after 23 days of exposure, while the other doses exhibited commensurate levels of transformation at much latter stages; 28 and 38 days of exposure to 30µM and 10µM of H₂O₂ respectively.
Figure 4.9: (A) Growth curve of CCD841CoN cells after exposure to hydrogen peroxide treatments at different concentrations. Morphology of cells at (B) 10µM, (C) 30µM, and (D) 50µM hydrogen peroxide treatments. Alteration to the cell growth pattern was accompanied by enlargement of CCD841CoN cells similar to IMR90 cells but formation of stress granules was not apparent in the former. Magnification, 200X. Data are represented as mean ± SD. n=3 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
In IMR90 cells, the divergence of 100nM doxorubicin growth curve relative to the other doses transpired after day 10 of treatment, while the divergence between 40nM and 70nM doxorubicin occurred after day 20 and onward (Figure 4.10A). The divergence amongst the growth curves indicate significant drop (P < 0.001) in cell population relative to the ascending curve(s). From day 21 and beyond, the 100nM doxorubicin growth curve on the graph was parallel to horizontal axis, indicating cessation of detectable cell population growth; whilst the inclining curves of the other doses have indicated still growing population of IMR90 cells albeit at a much reduced rate compared to the earlier days.

Accompanying the altered cell growth pattern was the overt changes to the morphological features of IMR90 cells such as size expansion and formation of stress granules (Figures 4.10B-D). The transformation was apparent on large majority (< 92%) of cells treated with 50µM H₂O₂ after 25 days of exposure while the other doses exhibited commensurate levels of transformation at much latter stages; 30 and 45 days of exposure to 30µM and 10µM of H₂O₂ respectively.
Figure 4.10: (A) Growth curve of IMR90 cells after exposure to doxorubicin treatments at different concentrations. Morphology of cells at (B) 40nM, (C) 70nM, and (D) 100nM doxorubicin treatments. Accompanying the altered cell growth pattern was the overt changes to the morphological features of IMR90 cells such as size expansion and formation of stress granules. Magnification, 200X. Data are represented as mean ± SD. n=3 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
In CCD841CoN cells, the divergence of 100nM doxorubicin growth curve relative to the other doses transpired after day 10 of treatment, while the divergence between 40nM and 70nM doxorubicin occurred after day 20 and onward (Figure 4.11A). The divergence amongst the growth curves indicates significant drop (P<0.001) in cell population relative to the ascending curve(s). From day 21 and beyond, the 100nM doxorubicin growth curve on the graph was parallel to horizontal axis, indicating cessation of detectable cell population growth; whilst the inclining curves of the other doses have indicated still growing population of IMR90 cell albeit at a much reduced rate compared to earlier days.

Alteration to the cell growth pattern was accompanied by enlargement of CCD841CoN cells similar to IMR90 cells but formation of stress granules was not apparent in the former (Figures 4.11B-D). The size increase was observed on large majority (<95%) of cells treated with 50µM H₂O₂ after 23 days of exposure, while the other doses exhibited commensurate levels of transformation at much latter stages; 28 and 38 days of exposure to 30µM and 10µM of H₂O₂ respectively.
Figure 4.11: (A) Growth curve of CCD841CoN cells after exposure to doxorubicin treatments at different concentrations. Morphology of cells at (B) 40nM, (C) 70nM, and (D) 100nM doxorubicin treatments. Alteration to the cell growth pattern was accompanied by enlargement of CCD841CoN cells similar to IMR90 cells but formation of stress granules was not apparent in the former. Magnification, 200X. Data are represented as mean ± SD. n=3 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.3.2(b) BrdU incorporation assay analysis

BrdU based cell proliferation assay was performed on the doxorubicin and H\textsubscript{2}O\textsubscript{2} treated cells on day 16. The percentage of cells that scored positive for BrdU incorporation was denoted by each column on the graph. Quintessentially, each column signifies the amount of cycling cells within its own population. Any treatment that limits the proliferative potential of the cells will reduce the total number of cells compared to untreated control. Suppression of cell proliferation indicates that the viable cells have entered cell cycle arrest, which is a hallmark of cellular senescence. In this experiment, proliferating cells falling below the threshold level of 10% were considered successful for the induction of premature senescence as described in Nogueira et al (Nogueira et al., 2008).

All the concentrations of H\textsubscript{2}O\textsubscript{2} had severely limited the cycling cells in the IMR90 populous and the difference between the treatments versus control were highly significant (P < 0.001) (Figure 4.12A). By day 16 of treatment with 50\textmu M H\textsubscript{2}O\textsubscript{2}, there was a reduction of 91% in cycling cells compared to untreated control (P < 0.001); whilst the cycling cell population of both 10\textmu M and 30\textmu M groups were suppressed at approximately 78% (P < 0.001) and 84% (P < 0.001) respectively compared to untreated control. 50\textmu M H\textsubscript{2}O\textsubscript{2} was substantially better at suppressing the expansion of IMR90 cells compared to the other doses. In 50\textmu M H\textsubscript{2}O\textsubscript{2} treated cells, the percentage of cycling cells were only 5%, hence 95% percent of the viable cells were in cell cycle arrest after 16 days of treatment. This was in stark contrast to the untreated control group whereby 94% of the cells in its
population were cycling. Thus, 50µM H₂O₂ treatment was highly effective in limiting the proliferation of IMR90 cells.

All the concentrations of H₂O₂ had severely limited the cycling cells in the CCD841CoN populous and the difference between the treatments versus control were highly significant (P < 0.001) (Figure 4.12B). By day 16 of treatment with 50µM H₂O₂, there was a reduction of 89.33% in cycling cells compared to untreated control (P < 0.001); whilst the cycling cell population of both 10µM and 30µM groups were suppressed at approximately 75% (P < 0.001) and 81% (P < 0.001) respectively compared to untreated control. 50µM H₂O₂ was substantially better at suppressing the expansion of CCD841CoN cells compared to the other doses. In 50µM H₂O₂ treated cells, the percentage of cycling cells were only 5.3%, hence 94.7% percent of the viable cells were in cell cycle arrest after 16 days of treatment. This was in stark contrast to the untreated control group whereby 93% of the cells in its population were cycling. Thus, 50µM H₂O₂ treatment was highly effective in limiting the proliferation of CCD841CoN cells.

The proliferation of IMR90 and CCD8411CoN cells were inhibited by H₂O₂ in a dose dependent manner in the order of 50µM >30µM >10µM compared to untreated control. 50µM H₂O₂ was substantially better at suppressing the expansion of both cells compared to the other doses. On day 16 after 50µM H₂O₂ treatment, the percentage of proliferating cells were only 5.0% for IMR90 and 5.3% for CCD8411CoN; thus adhering to the previously set threshold of below 10% cell proliferation limit. Hence, oxidative stress mediated by H₂O₂ was effective at suppressing the proliferation of both IMR90 and CCD8411CoN cells.
Figure 4.12: Quantification of proliferating cells in (A) IMR90 and (B) CCD841CoN cultures after treatment with different concentrations of hydrogen peroxide. 5-Bromo-2’-deoxyuridine (BrdU) based cell proliferation in the cultures were detected by immunofluorescence. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
All the concentrations of doxorubicin had severely limited the cycling cells in the IMR90 populous and the difference between the treatments versus control were highly significant (P < 0.001) (Figure 4.13A). By day 16 of treatment with 100nM doxorubicin, there was a reduction of 91% in cycling cells compared to untreated control (P < 0.001); whilst the cycling cell population of both 40nM and 70nM groups were suppressed at approximately 78% (P < 0.001) and 84% (P < 0.001) respectively compared to untreated control. 100nM doxorubicin was substantially better at suppressing the expansion of IMR90 cells compared to the other doses. In 100nM doxorubicin treated cells, the percentage of cycling cells were only 4.6%, hence 95% percent of the viable cells were in cell cycle arrest after 16 days of treatment. This was in stark contrast to the untreated control group whereby 94% of the cells in its population were cycling. Thus, 100nM doxorubicin treatment was highly effective in limiting the proliferation of IMR90 cells.

All the concentrations of doxorubicin had severely limited the cycling cells in the CCD841CoN populous and the difference between the treatments versus control were highly significant (P < 0.001) (Figure 4.13B). By day 16 of treatment with 100nM doxorubicin, there was a reduction of 91% in cycling cells compared to untreated control (P < 0.001); whilst the cycling cell population of both 40nM and 70nM groups were suppressed at approximately 74% (P < 0.001) and 83.67% (P < 0.001) respectively compared to untreated control. 100nM doxorubicin was substantially better at suppressing the expansion of CCD841CoN cells compared to the other doses. In 100nM doxorubicin treated cells, the percentage of cycling cells were only 7.58%, hence 92% percent of the viable cells were in cell cycle arrest after 16 days of treatment. This was in stark contrast
to the untreated control group whereby 93.5% of the cells in its population were cycling. Thus, 100nM doxorubicin treatment was highly effective in limiting the proliferation of CCD841CoN cells.

The proliferation of IMR90 and CCD8411CoN cells were inhibited by doxorubicin in a dose dependent manner in the order of 100nM >70nM >40nM compared to untreated control. 100nM doxorubicin was substantially better at suppressing the expansion of both cells compared to the other doses. On day 16 after 100nM doxorubicin treatment, the percentage of proliferating cells were only 4.6% for IMR90 and 7.58% for CCD8411CoN; thus adhering to the previously set threshold of below 10% cell proliferation limit. Hence, genotoxic stress mediated by doxorubicin was effective at suppressing the proliferation of both IMR90 and CCD8411CoN cells.
Figure 4.13: Quantification of proliferating cells in (A) IMR90 and (B) CCD841CoN cultures after treatment with different concentrations of doxorubicin. 5-Bromo-2’-deoxyuridine (BrdU) based cell proliferation in the cultures were detected by immunofluorescence. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.3.2(c) Senescence associated-β-galactosidase assay analysis

Cell senescence assay for the detection of SA-β-galactosidase was performed on the doxorubicin and H₂O₂ treated cells on day 16 post treatment. This test directly measures the amount of senescent cells that are expressing the enzyme β-galactosidase at pH 6. As the cells were induced to undergo senescence, they will express higher levels of this enzyme and consequently increasing the level of positive staining observable within the cell population. The percentage of cells that scored positive for SA-β-galactosidase staining was denoted by each column on the graph. Quintessentially, each column signifies the amount of senescent cells within its own population. In this experiment, threshold level exceeding 80% of SA-β-galactosidase expression within the cell population were considered successful for the induction of premature senescence as described in Dimri et al (Dimri et al., 1995).

All the concentrations of H₂O₂ had induced cellular senescence in the IMR90 populous and the difference between the treatments versus control were highly significant (P < 0.05 and P < 0.001) (Figure 4.14A). By day 16 of treatment with 50µM H₂O₂, there was an 81% increase in the expression of SA-β-galactosidase compared to control (P < 0.001); whilst for the 10µM and 30µM groups, the expression of SA-β-galactosidase increased by 16.67% (P < 0.05) and 56.33% (P < 0.001) respectively compared to untreated control. 50µM H₂O₂ was substantially better at inducing premature senescence on IMR90 cells compared to the other doses. More than 92% of the cells within its population were senescent compared to the low population of senescent cells spotted in the control group which was less than 12%. 

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All the concentrations of H₂O₂ had induced cellular senescence in the CCD841CoN populous and the difference between the treatments versus control were highly significant (P < 0.001) (Figure 4.14B). By day 16 of treatment with 50µM H₂O₂, there was a 77% increase in the expression of SA-β-galactosidase compared to control (P < 0.001); whilst for the 10µM and 30µM groups, the expression of SA-β-galactosidase increased by 31% (P < 0.001) and 60.67% (P < 0.001) respectively compared to untreated control. 50µM H₂O₂ was substantially better at inducing premature senescence on CCD841CoN cells compared to the other doses. More than 90% of the cells within its population were senescent compared to the low population of senescent cells spotted in the control group which was less than 11%.

The senescence induction of IMR90 and CCD8411CoN cells by H₂O₂ occurred in a dose dependent manner in the order of 50µM >30µM >10µM compared to untreated control. 50µM H₂O₂ was substantially better at inducing premature senescence on both cells compared to the other doses. On day 16 after 50µM H₂O₂ treatment, the percentage of SA-β-galactosidase expressing cells were 92% for IMR90 and 90% for CCD8411CoN; thus adhering to the previously set threshold of above 80% SA-β-galactosidase expression limit. Hence, oxidative stress mediated by H₂O₂ was effective at inducing premature senescence on IMR90 and CCD8411CoN cells.
Figure 4.14: Quantification of SA-β-galactosidase expression in (A) IMR90 and (B) CCD841CoN cells after treatment with different concentrations of hydrogen peroxide. SA-β-galactosidase activity in the cultures were cytochemically detected at pH 6. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
All the concentrations of doxorubicin had induced cellular senescence in the IMR90 populous and the difference between the treatments versus control were highly significant (P < 0.01 and P < 0.001) (Figure 4.15A). By day 16 of treatment with 100nM doxorubicin, there was an 83% increase in the expression of SA-β-galactosidase compared to control (P < 0.001); whilst for the 40nM and 70nM groups, the expression of SA-β-galactosidase increased by 14% (P < 0.01) and 55.33% (P < 0.001) respectively compared to untreated control. 100nM doxorubicin was substantially better at inducing premature senescence of IMR90 cells compared to the other doses. More than 95% of the cells within its population were senescent compared to the low population of senescent cells spotted in the control group which was less than 13%.

All the concentrations of doxorubicin had induced cellular senescence in the CCD841CoN populous and the difference between the treatments versus control were highly significant (P < 0.001) (Figure 4.15B). By day 16 of treatment with 100nM doxorubicin, there was a 77.33% increase in the expression of SA-β-galactosidase compared to control (P < 0.001); whilst for the 40nM and 70nM groups, the expression of SA-β-galactosidase increased by 29% (P < 0.001) and 55% (P < 0.001) respectively compared to untreated control. 100nM doxorubicin was substantially better at inducing premature senescence of CCD841CoN cells compared to the other doses. More than 91% of the cells within its population were senescent compared to the low population of senescent cells spotted in the control group which was less than 14%.
The senescence induction of IMR90 and CCD8411CoN cells by doxorubicin occurred in a dose dependent manner in the order of 100nM >70nM >40nM compared to untreated control. 100nM doxorubicin was substantially better at inducing premature senescence on both cells compared to the other doses. On day 16 after 100nM doxorubicin treatment, the percentages of SA-β-galactosidase expressing cells were 95% for IMR90 and 91% for CCD8411CoN; thus adhering to the previously set threshold of above 80% SA-β-galactosidase expression limit. Hence, genotoxic stress mediated by doxorubicin was effective at inducing premature senescence on IMR90 and CCD8411CoN cells.
Figure 4.15: Quantification of SA-β-galactosidase expression in (A) IMR90 and (B) CCD841CoN cells after treatment with different concentrations of doxorubicin. SA-β-galactosidase activity in the cultures were cytochemically detected at pH 6. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.3.2(d) SAHF assay analysis

Cell senescence assay for the detection of SAHF was performed on the doxorubicin and H₂O₂ treated cells on day 16 post treatment. This test measures the amount of senescent cells present in the cell population based on the formation of SAHF that is detectable using nuclear stain, Hoechst. As the cells are induced to undergo senescence, SAHF formation will increase within the cell population. The percentage of cells that scored positive for SAHF formation was denoted by each column on the graph. Quintessentially, each column signifies the amount of senescent cells within its own population. In this experiment, threshold level exceeding 80% of SAHF formation within the cell population were considered successful for the induction of premature senescence as described in Soliman et al (Soliman et al., 2008).

All the concentrations of H₂O₂ had induced cellular senescence in the IMR90 populous and the difference between the treatments versus control were highly significant (P < 0.01 and P < 0.001) (Figure 4.16A). By day 16 of treatment with 50µM H₂O₂, there was a 73% increase in the SAHF formation compared to control (P < 0.01); whilst for the 10µM and 30µM groups, formation of SAHF increased by 20% (P < 0.001) and 57.67% (P < 0.001) respectively compared to untreated control. 50µM H₂O₂ was substantially better at inducing premature senescence on IMR90 cells compared to the other doses. More than 86% of the cells within its population were senescent compared to the low population of senescent cells spotted in the control group that was less than 13%. 
All the concentrations of H$_2$O$_2$ had induced cellular senescence in the CCD841CoN populous and the difference between the treatments versus control were highly significant (P < 0.05 and P < 0.001) (Figure 4.16B). By day 16 of treatment with 50µM H$_2$O$_2$, there was a 71.33% increase in the SAHF formation compared to control (P < 0.001); whilst for the 10µM and 30µM groups, formation of SAHF increased by 13.67% (P < 0.05) and 52.33% (P < 0.001) respectively compared to untreated control. 50µM H$_2$O$_2$ was substantially better at inducing premature senescence of CCD841CoN cells compared to the other doses. More than 84% of the cells within its population were senescent compared to the low population of senescent cells spotted in the control group that was less than 12.66%.

The senescence induction of IMR90 and CCD8411CoN cells by H$_2$O$_2$ occurred in a dose dependent manner in the order of 50µM >30µM >10µM compared to untreated control. 50µM H$_2$O$_2$ was substantially better at inducing premature senescence of both cells compared to the other doses. On day 16 after 50µM H$_2$O$_2$ treatment, the percentage of SAHF formation were 86% for IMR90 and 84% for CCD8411CoN; thus adhering to the previously set threshold of above 80% SAHF formation limit. Hence, oxidative stress mediated by H$_2$O$_2$ was effective at inducing premature senescence on IMR90 and CCD8411CoN cells.
Figure 4.16: Quantification of SAHF formation in (A) IMR90 and (B) CCD841CoN cells after treatment with different concentrations of hydrogen peroxide. SAHF formation in cultures were detected by Hoechst 33342 nuclear staining. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
All the concentrations of doxorubicin had induced cellular senescence in the IMR90 populous and the difference between the treatments versus control were highly significant (P < 0.01 and P < 0.001) (Figure 4.17A). By day 16 of treatment with 100nM doxorubicin, there was a 72.67% increase in the formation of SAHF compared to control (P < 0.001); whilst for the 40nM and 70nM groups, formation of SAHF increased by 21.67% (P < 0.01) and 56% (P < 0.001) respectively compared to untreated control. 100nM doxorubicin was substantially better at inducing premature senescence on IMR90 cells compared to the other doses. More than 84% of the cells within its population were senescent compared to the low population of senescent cells spotted in the control group that was less than 12%.

All the concentrations of doxorubicin had induced cellular senescence in the CCD841CoN populous and the difference between the treatments versus control were highly significant (P < 0.01 and P < 0.001) (Figure 4.17B). By day 16 of treatment with 100nM doxorubicin, there was a 71.33% increase in the formation of SAHF compared to control (P < 0.001); whilst for the 40nM and 70nM groups, formation of SAHF increased by 15% (P < 0.01) and 55.67% (P < 0.001) respectively compared to untreated control. 100nM doxorubicin was substantially better at inducing premature senescence on CCD841CoN cells compared to the other doses. More than 81% of the cells within its population were senescent compared to the low population of senescent cells spotted in the control group that was less than 11%.
The senescence induction of IMR90 and CCD8411CoN cells by doxorubicin occurred in a dose dependent manner with 100nM >70nM >40nM compared to untreated control. 100nM doxorubicin was substantially better at inducing premature senescence on both cells compared to the other doses. On day 16 after 100nM doxorubicin treatment, the percentage of SAHF formation were 84% for IMR90 and 81% for CCD8411CoN; thus adhering to the previously set threshold of above 80% SAHF formation limit. Hence, genotoxic stress mediated by doxorubicin was effective at inducing premature senescence on IMR90 and CCD8411CoN cells.
**Figure 4.17:** Quantification of SAHF formation in (A) IMR90 and (B) CCD841CoN cells after treatment with different concentrations of doxorubicin. SAHF formation in cultures were detected by Hoechst 33342 nuclear staining. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.3.3 Comparative analysis of cell ageing methods

To evaluate the effectiveness of cell ageing methods used in this study, the results of biochemical assays were comparatively analyzed. IMR90 and CCD8411CoN cells were able to reach hayflick limit by replicative senescence at PDL 55 and PDL 48, respectively. IMR90 and CCD8411CoN cells at this population doubling levels adhered to the senescent cell benchmarks in accordance with the specific threshold levels established for each biochemical experiment. Both H$_2$O$_2$ and doxorubicin were able to induce premature senescence on IMR90 and CCD8411CoN cells at varying degrees depending on the dosage of the treatment. However, 100nM doxorubicin and 50µM H$_2$O$_2$ treatments were able to successfully induce premature senescence on IMR90 and CCD8411CoN cells after 16 days of ageing based upon the specific threshold levels established for each biochemical experiment. The results from different cell senescence induction methods are summarized in Table 4.1.

By comparing the data of stress induced premature senescence to the one obtained from replicative senescence, no incongruity was inferred with regards to senescence induction proficiency. In summary, the difference between young (control) and senescent cells were highly significant (P < 0.001) in cell proliferation (Figure 4.18), SA-β-galactosidase expression (Figure 4.19), and SAHF formation (Figure 4.20) assays, while the difference amongst the senescent cells were not statistically significant. Thus, H$_2$O$_2$ and doxorubicin methods were used in conjunction with replicative senescence for cell ageing.
Table 4.1: Comparison of data from different cell senescence induction methods.

Data are represented as mean ± SD. n = 6 for each group.

<table>
<thead>
<tr>
<th>Ageing methods</th>
<th>Cell Proliferation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SA-β-Galactosidase Expression&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SAHF Formation&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IMR90 (%)</td>
<td>CC8411CoN (%)</td>
<td>IMR90 (%)</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;i&lt;/sup&gt;</td>
<td>5.0 ± 0.38</td>
<td>92.3 ± 2.33</td>
<td>86.0 ± 2.10</td>
</tr>
<tr>
<td>Doxorubicin&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>4.6 ± 0.25</td>
<td>95.0 ± 4.25</td>
<td>84.0 ± 4.15</td>
</tr>
<tr>
<td>Replication&lt;sup&gt;iii&lt;/sup&gt;</td>
<td>Senescence&lt;sup&gt;iii&lt;/sup&gt;</td>
<td>9.0 ± 1.50</td>
<td>92.3 ± 3.15</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>8.0 ± 1.85</td>
<td>92.0 ± 4.58</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Cell proliferation below the threshold level of 10% is considered successful for the induction of premature senescence.

<sup>b</sup>: SA-β-galactosidase expression exceeding threshold level of 80% is considered successful for the induction of premature senescence.

<sup>c</sup>: SAHF formation exceeding threshold level of 80% is considered successful for the induction of premature senescence.

<sup>i</sup>: Data for senescence induction on IMR90 and CC8411CoN cells using 50µM of H<sub>2</sub>O<sub>2</sub>.

<sup>ii</sup>: Data for senescence induction on IMR90 and CC8411CoN cells using 100nM of doxorubicin.

<sup>iii</sup>: Data for PDL 55 for IMR90 cells and PDL 48 for CC8411CoN cells.
Figure 4.18: Effects of senescence induction on cell proliferation. Fluorescence micrograph of (A) IMR90 cells, (B) CCD841CoN cells and (C) quantification of cell proliferation. 5-Bromo-2’-deoxyuridine (BrdU) based cell proliferation in the cultures were detected by immunofluorescence. Magnification, 100X. Control: young IMR90 and CCD841CoN cells, RS: replicative senescence, H$_2$O$_2$: hydrogen peroxide, DOX: doxorubicin. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Continued…….
Figure 4.19: Effects of senescence induction on SA-β-galactosidase expression. Bright field micrograph of (A) IMR90 cells, (B) CCD841CoN cells and (C) quantification of SA-β-galactosidase expression. SA-β-galactosidase activity in the cultures were cytochemically detected at pH 6. Scale bar = 100µm. Control: young IMR90 and CCD841CoN cells, RS: replicative senescence, H₂O₂: hydrogen peroxide, DOX: doxorubicin. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Figure 4.20: Effects of senescence induction on SAHF formation. Fluorescence micrograph of (A) IMR90 cells, (B) CCD841CoN cells and (C) quantification of SAHF formation. SAHF formation in cultures were detected by Hoechst 33342 nuclear staining. Magnification, 400X. Control: young IMR90 and CCD841CoN cells, RS: replicative senescence, H$_2$O$_2$: hydrogen peroxide, DOX: doxorubicin. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.3.4 Ageing biomarker analysis in primary fibroblast cells

To determine the correlation between replicative/premature senescence and chronological lifespan, senescence biomarker assays were performed on primary fibroblast cells of different age groups: AG04450 fetal human lung fibroblasts (12 weeks), AG02603 adult human lung fibroblasts (35 years) and AG02262 adult human lung fibroblasts (61 years). To establish proper controls for this experiment, young and senescent IMR90 cells were used as negative and positive controls, respectively. It was hypothesized that the results of the senescence biomarker assays would pan out as follows: (1) AG04450 and young IMR90 cells would have parallel results as both cells were derived from fetal human lung fibroblasts, (2) results of AG02603 would reveal intermediary disposition between the young and senescent IMR90 cells, and (3) AG02262 and senescent IMR90 cells would have parallel results due to age related cellular changes. However, the primary lung fibroblast cultures established from donors of divergent age groups did not reveal significant difference in cell proliferation (Figure 4.21), SA-ß-galactosidase expression (Figure 4.22), and SAHF formation (Figure 4.23) amongst the cell types. AG02603 and AG02262 cells did not show any characteristics of senescence phenotype based on the cell ageing biomarker results, including the more commonly observed changes such as enlargement and flattening of cells. Even though it can be argued that SA-ß-gal and SAHF staining did not reflect the chronological age of the donor or that the staining was cell type specific; cell cycle arrest which is the most prominent and incontrovertible characteristic of cellular senescence was not exhibited by AG02603 and AG02262 cells. Therefore, this argument can be dismissed and it was concluded that chronological age of cultures does not correlate with \textit{in vitro} induced senescent cultures.
Figure 4.21: (A) Fluorescence micrograph and (B) quantification of cell proliferation in primary fibroblast cells. 5-Bromo-2’-deoxyuridine (BrdU) based cell proliferation in the cultures were detected by immunofluorescence. Magnification, 100X. (–ve) control: young IMR90 cells; (+ve) control: senescent IMR90 cells. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Figure 4.22: (A) Bright field micrograph and (B) quantification of SA-β-galactosidase expression in primary fibroblast cells. SA-β-galactosidase activity in the cultures were cytochemically detected at pH 6. Scale bar = 50µm. (–ve) control: young IMR90 cells; (+ve) control: senescent IMR90 cells. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Figure 4.23: (A) Fluorescence micrograph and (B) quantification of SAHF formation in primary fibroblast cells. SAHF formation in cultures were detected by Hoechst 33342 nuclear staining. Magnification, 400X. (–ve) control: young IMR90 cells; (+ve) control: senescent IMR90 cells. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
4.4 Conclusion

The results have shown that senescence induction on IMR90 and CCD841CoN cells were successfully accomplished by the ageing methods employed in the current study. The difference amongst the ageing methods were insignificant in terms of effectiveness, however the time interval for consummation was expectedly longer in replicative senescence due to the propagation of cells. Verification of senescent phenotype was done through biochemical characterizations and based on the established benchmarks in the current experiment, senescent models for fibroblast and epithelial cells were successfully developed. Conversely, the results of senescence biomarker assays performed on primary fibroblast cells of different age groups did not show significant variance, indicating that chronological age of cultures may not necessarily correlate with in vitro induced senescent cultures.
5.1 Introduction

Visualization of the interaction between cells and nanoparticles is extremely useful in revealing occult details such as nanoparticle internalization by cells, its intracellular trafficking, and finally its localization within the cells. Non-invasive imaging devices with high magnification and resolution such as transmission electron microscopy, confocal microscopy and fluorescence microscopy can be utilized for this purposes. Concomitantly, with the aid of selective staining to highlight cells or its specific internal structures, the aforementioned information can be obtained with greater sensitivity and clarity. In the current study, cells and its major organelles such as lysosomes and nucleus were differentially stained with specific fluorophores to observe their interaction with the quantum dots through fluorescence microscopic techniques. Additionally, chemical inhibitors were also employed to probe distinct aspects of the cellular internalization process.
5.2 Materials and methods

5.2.1 Energy dependent cell uptake assay

Energy dependent cell uptake were studied by lowering cellular metabolism via temperature regulation at 4°C and by cellular ATP depletion via sodium azide/2-deoxyglucose treatment. The medium from CCD8411CoN and IMR90 cultures were removed from the wells and cells were washed twice with PBS. Cell tracker green CMFDA (500 nM) labeling solution in cell culture medium were added per 35-mm dish and the cells were incubated at 37°C for 1 h. After incubation, the labelling solutions were removed and the cells were washed 2X with PBS for 2 minutes each. QD particles prepared in cell culture medium were added in the wells and incubated for 1-3 hours at 4°C. For ATP depletion experiment, the cultures were treated with 10 mM sodium azide and 50 Mm 2-deoxyglucose for an additional 30 minutes at 37°C post CMFDA labelling and prior to incubation with QD particles for 1-3 hours at 37°C. Control cells were not pre-treated with sodium azide/2-deoxyglucose and incubated with QD particles for 1-3 hours at 37°C. After incubation, the solutions were removed and cells were washed three times with PBS. Finally, Fresh PBS were added in all the wells and observed under fluorescence microscope at 400X magnification. Cells were identified at excitation/emission wavelength of 492/517 nm and the QDs were identified at excitation/emission wavelength of 640/705 nm. 100 cells were scored per treatment from 6 replicates of 3 independent experiments.
5.2.2 Endocytic cell uptake assay

Cell uptake via endocytosis were studied by using endocytotic inhibitors monodansylcadaverine (MDC) and nystatine that targets clathrin dependent pathway and caveolae dependent pathway, respectively. The medium from CCD8411CoN and IMR90 cultures were removed from the wells and cells were washed twice with PBS. Cell tracker green CMFDA (500 nM) labeling solution in cell culture medium were added per 35-mm dish and the cells were incubated at 37°C for 1 h. After incubation, the labelling solutions were removed and the cells were washed 2X with PBS for 2 minutes each. 75 μM MDC or 75 μM nystatine in cell culture medium were added per 35-mm dish and the cells were incubated at 37°C for 30 min. After incubation, the inhibitor solutions were removed and the cells were washed 2X with PBS for 2 minutes each. QD particles prepared in cell culture medium were added in the wells and incubated for 1-3 hours at 37°C. Control cells were not pre-treated with inhibitors and incubated with QD particles for 1-3 hours at 37°C. After incubation, the solutions were removed and cells were washed three times with PBS. Finally, Fresh PBS were added in all the wells and observed under fluorescence microscope at 400X magnification. Cells were identified at excitation/emission wavelength of 492/517 nm and the QDs were identified at excitation/emission wavelength of 640/705 nm. 100 cells were scored per treatment from 6 replicates of 3 independent experiments.
5.2.3 Exocytosis imaging

Exocytosis of QD by CCD8411CoN and IMR90 cells were investigated with fluorescence microscopy. The medium from CCD8411CoN and IMR90 cultures were removed from the wells and cells were washed twice with PBS. Cell tracker green CMFDA (500 nM) labeling solution in cell culture medium were added per 35-mm dish and the cells were incubated at 37°C for 1 h. After incubation, the labelling solutions were removed and the cells were washed 2X with PBS for 2 minutes each. QD particles prepared in cell culture medium were added in the wells and incubated for 1-4 hours at 37°C. At specific time intervals during incubation, cells were observed under fluorescence microscope at 400X magnification. Cells were identified at excitation/emission wavelength of 492/517 nm and the QDs were identified at excitation/emission wavelength of 640/705 nm. 100 cells were scored per treatment from 6 replicates of 3 independent experiments.

5.2.4 Colocalization imaging

Colocalization of QDs in lysosomes of CCD8411CoN and IMR90 cells were investigated with fluorescence microscopy. The medium from CCD8411CoN and IMR90 cultures were removed from the wells and cells were washed twice with PBS. LysoTracker blue (120 nM) labeling solution in cell culture medium were added per 35-mm dish and the cells were incubated at 37°C for 1 h. After incubation, the labelling solutions were removed and the cells were washed 2X with PBS for 2 minutes each. QD nanoparticles prepared in cell culture medium were added in the wells and incubated for 1-4 hours at
37°C. After incubation, the solutions were removed and cells were washed three times with PBS. Finally, Fresh PBS were added in all the wells and observed under fluorescence microscope at 400X magnification. Lysosomes were identified at excitation/emission wavelength of 370/422 nm and the QDs were identified at excitation/emission wavelength of 640/705 nm.

5.2.5 Statistical analyses

Statistical analysis was performed based on one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer post hoc test of significance by using Prism® 5.0 software (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered to be statistically significant.
5.3 Results and Discussion

5.3.1 Energy dependent cell uptake of nanoparticles

Cell uptake studies were performed to gain deeper understanding about the kinetics of the nanoparticles within the confines of in vitro culture system and its interaction with different cell types. 30nM QD-PEG and 45nM QD-HC were used in this study because their aggregation and gravitational sediment sizes in cultures were similar as explained earlier (section 3.3.4), thus eliminating the size dependent cellular uptake of nanoparticles as reported by other researchers (Rejman et al., 2004; Li et al., 2008).

Based on the results, it was shown that entry of the QDs into the cells occurred predominantly via endocytosis. In cell cultures that were cooled to 4 C°, the internalization of QDs were significantly halted (P < 0.001) compared to QDs that were incubated at 37 C° for 90 minutes (Figure 5.1). The percentage of internalization was less than 8% for 30nM QD-PEG and 45nM QD-HC in all the cell types. This occurrence can be attributed to the increased rigidity of plasma membranes and decreased metabolic activity of the cells at 4 C°; thus emphasizing the cell’s reliance on energy dependent mechanism to internalize the QDs. It was also anticipated that the aggregation and sedimentation process of the nanoparticles were affected by the decreased Brownian motion as a consequence of reduced thermal energy in cultures at 4 C°; thus, reducing its interaction and internalization by the cells at the bottom of the culture plates.
To understand the cellular internalization of the nanoparticles without affecting its aggregation and sedimentation process due to reduced thermal energy as stated prior, the cultures were pre-incubated with NaN₃ + 2DG, which is a chemical inhibitor of mitochondrial activity. In NaN₃ + 2DG pre-treated senescent cultures, internalization of QDs were significantly halted (P < 0.001) compared to normal cultures (37 C°, without NaN₃ + 2DG); thus conforming the energy dependent endocytic pathway of entry into the senescent cells.

There was no significant difference between the internalization of 30nM QD-PEG and 45nM QD-HC by the senescent cells. However, the internalization of 30nM QD-PEG and 45nM QD-HC in young cells were significantly higher (P < 0.001) compared to the senescent cells. One of the contributing factor for the lowered uptake of QDs in senescent cells would be due to the depression of its mitochondrial activity relative to the young cells. As cells undergo senescence, the process of mitochondrial fission is interrupted due to the accumulated mutation in mtDNA (Green et al., 2011). Over time, this will result in reduced mtDNA replication and subsequently distribution of its low copy number in newly formed daughter mitochondria.

Consequently, mitochondrion with low copy number of mtDNA will lose the ability to duplicate and appear to be abnormally large in size. This structural change occurs due to the large amount of mitochondrial proteins that were synthesized by the nuclear genome and transported to the defunct mitochondria will remain therein resulting in its
size expansion. These large mitochondria are less likely to be autophagocytosed and recycled by the cells, thus gradually deteriorating in function. In senescent cells, a large majority of mitochondria are in this defective state with diminished capacity to carry out respiratory function such as citric acid cycle, oxidative phosphorylation and ATP production. The attenuated endocytic capacities of senescent cells were further halted by treatment with NaN₃ + 2DG compared to the young cells.

The other contributing factor could be due to the hyporesponsiveness in transduction signaling of the senescent cells. The downregulation of amphiphysin-1 and upregulation of caveolin receptors were identified as the primary reasons for the functional decay in signaling mechanisms observed in senescent cells (Park et al., 2001). Dysfunctional signaling transduction will lead to impaired receptor mediated endocytosis. Signaling transduction requires expansion of energy in the form of ATP and in senescent cells with hyporesponsiveness to stimuli, the requirement for ATP will be even more to effectively modulate the signal from plasma membrane and then relay it downstream to initiate the endocytosis process. The ATP production in senescent cell cultures that were pretreated with NaN₃ + 2DG was already low due to the reasons stated prior. As such, the cumulative response of the senescent cells would be to delay the entry of QDs into the cells via the endocytic pathway.
**Figure 5.1:** Internalization of QDs in (A) IMR90 and (B) CCD841CoN cells cultured at different temperatures and endocytic inhibitors. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Another factor that could have contributed to the reduced internalization rate of senescent cells that were pre-incubated with NaN₃ + 2DG was its enlarged size relative to the young cells (Figure 5.2). It was reported that larger cells having greater spreading area in the cell culture flask have increased membrane tension (Wang et al., 2016). In the context of endocytosis, the membranes of larger cells require greater deformation energy to overcome membrane tension for the purpose of engulfing materials and forming endocytotic vesicles (Treuel et al., 2013; Zhang et al., 2015).

Similarly, larger senescent cells with increased spreading area in the culture flask will require higher energy expenditure to internalize the nanoparticles compared to the young cells. Pre-treatment of senescent cultures with NaN₃ + 2DG will exacerbate this deficiency by halting its ATP production and further diminishing its endocytic competency. Suffice to say, these combined factors should explain the lower internalization rate of QDs in senescent cells compared to the young cells.
Figure 5.2: Fluorescence micrographs of young and senescent (A) IMR90 cells and (B) CCD841CoN cells. The cells were stained with CMFDA CellTracker fluorescent dye. Bar = 50µm.
The internalization rate of QDs between the young and senescent cells in cultures incubated at 37 °C without any endocytic inhibitors were negligible. It was presumed that the increased cell size of senescent cells would have compensated for the reduction of its endocytic potential as discussed earlier. Increased cell size implies more exposure of the cell’s surface area in the culture, hence more accessible entry points for the QDs to be trafficked into the senescent cells albeit it’s slower internalization capacity (Wang et al., 2016). This was especially true for the IMR90 cells, as the ratio in size difference between the young and senescent cells were almost 1:50 (Figure 5.3). In CCD841CoN cells, there was no significant difference in the internalization rate of QDs between the young and senescent cells.

Based on the data, it was also indicated that the difference in mean percentage values of nanoparticle uptake between young and senescent CCD841CoN cells at normal culture conditions were slightly higher in comparison to the IMR90 cell pairs. In other words, the young CCD841CoN cells have more endocytic potential compared to its counterpart (senescent CCD841CoN cells) than the young IMR90 cells. This finding can be ascribed to the difference in cell size ratio between the young and senescent cells of each type. The cell size ratio between the young and senescent CCD841CoN cells were 1:10, which was much smaller compared to the ratio of young and senescent IMR90 cells at 1:50. As such, CCD841CoN senescent cells’ surface area were less exposed in the cultures relative to IMR90 senescent cells; thus, CCD841CoN young cells procuring an advantage with regards to the overall endocytic potential in the tradeoff between more accessible entry points in larger senescent cells versus more efficient uptake in smaller young cells.
Figure 5.3: Cell size of IMR90 and CCD841CoN cells at different states. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
5.3.2 Endocytic cell uptake of nanoparticles

To further elucidate the mechanism of nanoparticle internalization by endocytosis, experiments were undertaken by employing endocytotic inhibitors monodansylcadaverine (MDC) and nystatine that targets clathrin dependent pathway and caveolae dependent pathway, respectively. These two pathways are commonly utilized by the cells to internalize nanoparticles as reported by numerous studies and a distinguishing feature that separates their internalization process is the inherent size restriction in endocytosing nanoparticles (Rejman et al., 2004; Kelf et al., 2010). Particles within the size ranges of 50-500 nm was reported to be internalized by the cells via clathrin mediated endocytosis and larger nanoparticles in the ranges of 500-1000 nm was reported to be internalized via caveolae mediated endocytosis (Rejman et al., 2004; Li et al., 2008). Hence, it was postulated that the QDs in the present study were mainly internalized via clathrin-mediated pathway based on the measured size of nanoparticle clusters formed as a result of gravitational sedimentation (section 3.3.4).

To elaborate further, QDs in colloid will coalesce to form larger agglomerates after dispersion into cell culture medium, which will in turn increase its density and accelerate the settling down process onto the monolayer of cells at the bottom of the wells due to the action of gravitational forces. The piling up of individual agglomerates into larger clusters as they descend to the cell surface, constitutes the individual sediment sizes. These clusters of QDs or sediments represent the tangible sizes of QD that the cells encounter and potentially internalize as opposed to its primary or hydrodynamic sizes. As discussed previously (section 3.3.4), gravitational sedimentation analysis using AFM have confirmed that 90% of individual sediments formed for both 30nM QD-PEG and 45nM
QD-HC are below the size of 200 nm after 30 min of incubation in serum free media, thus favoring its internalization via clathrin mediated endocytosis.

From the results it was shown that MDC reduced the QD uptake by 65-70% in IMR90 and 75-80% in CCD841CoN cells, demonstrating that all the cells utilized clathrin dependent endocytosis as the primary mechanism to traffic in the QDs (Figure 5.4). The internalization of QDs were significantly lower (P < 0.001) in senescent cells compared to young cells in both cell types. The disparity in the internalization of QDs between the young and senescent cells was presumed to be a reflection on the attenuated clathrin dependent endocytotic activity of the latter due to downregulation of amphiphysin-1 proteins (Park et al., 2001; Park et al., 2002). Amphiphysin-1 plays a major role in clathrin-mediated pathway by its involvement in the assembly of endocytotic vesicles and the reduced expression level of this protein in senescent cells could result in protracted internalization process. By pre-treating the cells with MDC which acts as an inhibitor of clathrin mediated pathway through stabilization of clathrin proteins (Ivanov, 2008), the endocytotic activity in senescent cells were depressed to a larger degree compared to the young cells.

This theory was further substantiated when nystatin treated cells did not have noticeable effect on the cellular uptake compared to the control cells. Nystatin specifically disrupts the caveloe mediated pathway of cells by distorting the structure and function of the cholesterol-rich membrane domains without affecting the clathrin mediated pathway (Ivanov, 2008). As such, cells treated with nystatin would not interfere with the internalization of QDs if their route of entry was via clathrin mediated pathway.
Figure 5.4: Internalization of QDs in (A) IMR90 cells and (B) CCD841CoN cells pretreated with endocytic inhibitors, monodansylcadaverine (MDC) and nystatin that targets clathrin dependent pathway and caveolae dependent pathway, respectively. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
From fluorescence microscopy analysis, the internalized QDs in senescent cells appeared larger than the young cells when both cultures were pretreated with nystatin (Figure 5.5). Once again, this anomaly was attributed to the protracted internalization process in senescent cells, whereby the longer the QD sediments rests on the surface of plasma membrane before being endocytosed by the cells, the probability of other nanoparticle agglomerates arbitrarily heaping onto the existing sediment increases.

Consequently, the mean size of individual QD clusters on the senescent cells will increase and manifests as larger internalized nanoparticles compared to the young cells. Evidently this was shown to be the case by gravitational sedimentation analysis using AFM, whereby as nanoparticle incubation time was extended from 30 to 90 minutes, the individual sediment sizes have increased by 30% for 45nM QD-HC and 50% for 30nM QD-PEG. The size increase in QD sediments was grossly exaggerated in the AFM study because in typical culture conditions, the cells would have internalized the nanoparticles much earlier during the incubation period.
Figure 5.5: Fluorescence micrographs of QDs uptake in young and senescent IMR90 and CCD841CoN cells that were pretreated with nystatin. Experiments were carried out at 37°C for 1h. Cells were stained with CMFDA CellTracker fluorescent dye (green) and QD-PEG/QD-HC nanoparticles were marked in red. Bar = 50µm.
5.3.3 Exocytosis of nanoparticles

Exocytosis of QDs were analyzed by counting the number of cells that expelled the internalized QDs after 1.5-4 hours of incubation in both young and senescent cells. The current experimental approach of quantifying the cells rather than the amount of QDs that were expelled was much more reliable due to the final state of the exocytosed artifacts. For instance, the expelled QDs from young cells appeared to be structurally different from the senescent cells; similarly, there was slight structural distinction in the exocytosed QDs between the senescent cell types and between the young cell types. Aggregated QDs were released from the young IMR90 and CCD841CoN cells, though larger cluster of aggregated QDs were seen more frequently in contact-inhibited cells compared to replicating cells of both types (Figure 5.6). Because the contact-inhibited cells were at close proximity to each other, the concentration gradient of the exocytosed QDs at the periphery of these cells will be denser and the smaller QD aggregates will be thermodynamically driven to consolidate into larger clusters; hence the size difference between the aggregated QDs that were expelled from replicating and contact-inhibited young cells.

In IMR90 senescent cells, the exocytosed QDs emerged as elongated linear threadlike structures that stretched away from the cells while still anchored to it and cross-linked with other similar structures from the neighboring cells (Figure 5.7). In the senescent CCD841CoN cells, the exocytosed QDs emerged as short linear threadlike structures that coil back into an entangled string configuration after it was detached from the cells (Figure 5.8). QDs that were exocytosed from all the cells were initially trapped
in the lysosomes prior to excretion. The exocytosis process appeared to be both cell type and cell state dependent even though explicit discernment between the exocytosed QD-PEG and QD-HC had not been made in this study.

**Figure 5.6:** Fluorescence micrographs of QDs exocytosis in (A) replicating IMR90 cells, (B) replicating CCD841CoN cells, (C) contact-inhibited IMR90 cells, and (D) contact inhibited CCD841CoN cells. Circles in the images represent exocytosed QD-PEG/QD-HC nanoparticles. Cells were stained with CMFDA CellTracker fluorescent dye (green) and QD-PEG/QD-HC nanoparticles were marked in red. Bar = 50µm.
Figure 5.7: Fluorescence micrographs of QDs exocytosis from senescent IMR90 cells in a time course experiment. White arrowheads point to exocytosed QD-PEG/QD-HC nanoparticles from the cells and micrographs are ordered from top to bottom to correspond with the progression of incubation time. Experiments were carried out at 37°C for 4h. Cells were stained with CMFDA CellTracker fluorescent dye (green) and QD-PEG/QD-HC nanoparticles were marked in red. Bar = 50µm.
**Figure 5.8:** Fluorescence micrographs of QDs exocytosis from senescent CCD841CoN cells in a time course experiment. White arrowheads point to exocytosed QD-PEG/QD-HC nanoparticles from the cells and micrographs are ordered from top to bottom to correspond with the progression of incubation time. Experiments were carried out at 37°C for 4h. Cells were stained with CMFDA. CellTracker fluorescent dye (green) and QD-PEG/QD-HC nanoparticles were marked in red. Bar = 50µm.
Approximately 92-95% of the young CCD841CoN cells and 88-90% of young IMR90 cells have exocytosed the internalized QDs within 1.5h of incubation period (Figure 5.9A). 30nM QD-HC was exocytosed at a much faster rate compared to 30nM QD-PEG or 45nM QD-HC by the all the young cells; whereby a large majority of the QDs were exocytosed within 40-45 minutes in IMR90 cells and 30-33 minutes in CCD841CoN cells. It was hypothesized that due to the smaller hydrodynamic diameter and reduced sediment sizes, 30nM QD-HC was internalized much earlier and subsequently exocytosed more rapidly by the young cells compared to 30nM QD-PEG or 45nM QD-HC. Hence, the current results were in agreement with prior studies that have reported on the size dependent exocytosis of cells and more specifically on smaller sized nanoparticles being exocytosed at a faster rate compared to the larger sized nanoparticles (Jin et al., 2009; Canton and Battaglia, 2012).

Similarly, the results have shown that 30nM QD-HC was internalized much earlier and subsequently exocytosed more rapidly by the senescent cells of both types compared to 30nM QD-PEG or 45nM QD-HC (Figure 5.9B). However, the exocytosis process in senescent cells took longer and only a small percentage of the cells were able to exocytose the QDs in the same time interval as the young cells. Approximately 60-65% of the senescent CCD841CoN cells and 45-50% of senescent IMR90 cells have exocytosed the internalized 30nM QD-HC within 4 hours of incubation period. At the same time interval, 10-12% of the senescent CCD841CoN cells and 14-17% of the senescent IMR90 cells were able to exocytose 45nM QD-HC; whilst 4-7% of the senescent CCD841CoN cells and 6-9% of the senescent IMR90 cells were able to exocytose 30nM QD-PEG.
It was conjectured, that the toxic effects induced by the internalized QDs would have interfered with the exocytosis process, resulting in its poor elimination from the senescent cells. The cytotoxicity data have shown that within 4 hours of incubation period, inhibition of senescent cells were already taking place due to the exposure of QDs, though the degree of the toxic effect varied among the QD types as a function of time (section 6.3.4). Inhibition of the cells increased in the order of 30nM QD-HC < 45nM QD-HC < 30nM QD-PEG; whilst exocytosis of the nanoparticles increased in the order of 30nM QD-PEG < 45nM QD-HC < 30nM QD-HC.

Therefore, an inverse correlation between exocytosis and cytotoxicity of QDs can be established. Even though the existing data does not lend to further elaboration, two other possibilities cannot be ruled out: (1) attenuated exocytotic activity had increased the susceptibility of senescent cells to the QDs’ toxic effects owing to its role as a detoxifying mechanism by which the intracellular QDs were expelled to reduce the localized nanoparticle concentration effect in lysosomes, and (2) minor interference in the exocytosis process due to QDs’ toxicity had set a retrograde feedback cascade which amplified the negative effects; thus, further debilitating the exocytosis process and the ensuing mutually destructive cycle had crippled the cellular functions.
Figure 5.9: (A) Exocytosis of QDs from young IMR90 and CCD841CoN cells after 1.5 hours of incubation. (B) Exocytosis of QDs from senescent IMR90 and CCD841CoN cells after 4 hours of incubation. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
5.3.4 Surface specific cell uptake of nanoparticles

The results have shown that the internalization of 45nM QD-HC were significantly higher (P < 0.001) compared to 30nM QD-PEG in all the cell types at 37 °C without any endocytic inhibitors; thus demonstrating that the internalization process was primarily particle type dependent. It was presumed that the internalization of anionic QD-HC will be lower than non-ionic QD-PEG due to repulsive electrostatic interaction with the negatively charged plasma membranes, yet the results have shown otherwise. Even though, having electrostatically neutral surface charge might increase the probability of QD-PEG’s adherence onto the surface of plasma membrane due to the absence of repelling forces compared to QD-HC, it may not necessarily translate into higher internalization by the cells. Many studies have linked surface charge of nanoparticles as an important factor in the adherence and/or internalization of nanoparticles into the cells (Chung et al., 2007; Frohlich, 2012). So far, the role of nanoparticle’s size as a factor in the cellular internalization process was relegated in the current study, however there was also a need to dispel nanoparticle’s surface charge as a factor that can influence the internalization process.

30nM QD-PEG has a surface charge of -2.0 mV, whereas 45nM QD-HC has a surface charge of -10.0 mV in the experimental conditions. If the surface charge of both QDs could be normalized at experimental conditions, then it is possible to determine whether the specific proteins/peptides present in the hard corona of QD-HC had favorably facilitated its internalization process. To achieve this purpose, the cell uptake experiments were carried out in cultures supplemented with 15% FBS. Protein adsorption onto the QDs
have imparted negative charges on its surface at a constant value as evidenced by the zeta potential results (section 3.3.3). Similarly, adherence of proteins/peptides from FBS will lower the net negative surface charge of QD-PEG, thereby accounting for the deficit surface charge that exists between 30nM QD-PEG and 45nM QD-HC.

Another crucial element that must be reckoned with before undertaking the QD’s surface charge normalization step is the fact that protein adsorption will also modulate the colloidal properties of 30nM QD-PEG. Therefore, to limit the discrepancies in the experiment as a consequence of these changes, internalization of 30nM QD-PEG was compared against 30nM QD-HC instead of 45nM QD-HC. 30nM QD-HC was essentially 30nM QD-PEG with hard protein corona from human plasma attached to it; thus it should mimic the colloidal behavior of 30nM QD-PEG when it interacts with proteins/peptides of FBS in the culture system.

FBS contains many growth factor, proteins, and peptides that are commonly added into the cell culture formulation for in vitro cell growth and proliferation. However, many researchers have reported that cellular internalization of various nanoparticles were restricted in cell cultures that were supplemented with FBS (Zhu et al., 2009; Patel et al., 2010; Tedja et al., 2012b). Since FBS was comprised of a complex mixture of proteins, adsorption of a small subgroup of this molecules onto the QD’s surface may inhibit its uptake by the cells of which fetuin was reported to be a major one FBS (Brown et al., 1992). In the current experiment, hard protein corona of QD-HC was derived from human
blood plasma in which fetuin was largely absent contrary to its high abundance in FBS (Brown et al., 1992).

The internalization of 30nM QD-PEG and 30nM QD-HC was highly constrained in all the cells that were cultured with 15% FBS supplementation relative to the cells without FBS (Figure 5.10). A plausible explanation was that the cell’s surface receptors were saturated by competing proteins from FBS, which restricted its endocytosis of the QDs. However, the result had shown significant difference (P < 0.001) in the internalization rate between 30nM QD-PEG and 30nM QD-HC. 30nM QD-PEG was restricted by 75-80% in both cell types cultured with FBS compared to cultures without FBS; whilst 30nM QD-HC was restricted by 60-70% in both cell types cultured with FBS compared to cultures without FBS. If the assumption that cell surface receptor saturation by FBS was true, then there wouldn’t be any disparity in the internalization rate between 30nM QD-PEG and 30nM QD-HC, thus invalidating this theory. A more convincing explanation would be that, FBS protein adsorption onto the QD’s surface had restricted its uptake by the cells.

In the case of QD-HC, the ensuing weak protein-protein interactions between the hard protein corona shell and FBS proteins in the cell culture medium would have shielded the protein corona’s epitopes that had facilitated QD-HC’s trafficking into the cells via receptor mediated endocytosis. SDS-PAGE results have shown that QD-HC nanoparticles that were incubated in cell culture medium with FBS, did not show any alteration to its
hard protein corona composition after the washing steps to remove the weakly adsorbed soft protein corona; thus dismissing the possibility of protein desorption or new protein formation within the hard corona mosaic (section 3.3.2). Similarly, DLS results based on QD-HC’s incubation in pre-conditioned medium containing proteins and cell debris have shown that the particle size distribution did not change noticeably due to aggregation/agglomeration as a consequence of these external interferences; thus, signifying the robustness of its colloidal properties within the cell culture system (section 3.3.3).

Without evidence of hard protein corona alteration or changes to the colloidal properties of QD-HC, it can be concluded that its internalization by the cells was restricted due to the masking of its epitopes by FBS’s soft protein corona and this protein-protein interaction occurs stochastically. The significance of soft protein corona in influencing the internalization of QD-HC was important in this particular experiment, yet it does not lend to deeper analysis outside the in situ process due to the complex and dynamic nature of these interactions.

In the case of QD-PEG, in situ adsorption of FBS proteins would alter its physiochemical characteristics and consequently its colloidal properties in the cell culture medium. This process was well documented in the previous section with the transformation of QD-PEG to QD-HC (section 3.3.2 - 3.3.3). In culture medium that were supplemented with 15% FBS, 30nM PEG and 30nM QD-HC ought to have similar
colloidal properties and surface charges, however the adsorbed protein/peptides on QD-PEG were exclusively formed by the components present only in FBS, hence it may have distinct protein corona profile from QD-HC. With regards to the internalization of QD-PEG by the cells, protein adsorption would have increased its dispersibility in the culture media, resulting in reduced agglomeration and gravitational sedimentation of QD-PEG; thus limiting its availability at the cell surface receptors for internalization. The internalization of QD-PEG could have been further impaired by the presence of inhibitory protein corona as discussed previously. Due to these factors, the internalization of QD-PEG was significantly reduced (P < 0.001) compared to the culture system devoid of FBS supplementation.

The primary objective of this experiment was to determine if the hard protein corona of QD-HC was solely responsible for its increased internalization by the cells compared to QD-PEG. The results have shown significant difference (P < 0.001) in the cellular uptake with 30nM QD-HC having the advantage over 30nM QD-PEG by 30-33% for IMR90 cells and 23-25% for CCD841CoN cells. Hence, it has been firmly established that hard protein corona of QD-HC had promoted its internalization and the absence of it on QD-PEG had rendered its internalization less effective by comparison. The percentage difference in the uptake between 30nM PEG and 30nM QD-HC in cultures supplemented with 15% FBS was much higher compared to 30nM PEG and 45nM QD-HC in cultures without FBS for both cell types. This implies that QD’s surface charge may have also influenced the internalization of nanoparticles in this experiment with neutrally charged nanoparticles having increased cell uptake compared to the negatively charged
counterpart. However, the evidence for this was not conclusive due to the nature of the experimental setup and should be taken anecdotally.

The results have consistently shown that the internalization of QD-HC was favored over QD-PEG in cultures that were supplemented with or without FBS. Detailed analysis of the hard protein corona by SDS-PAGE, MALDI-TOF/TOF and LC-MS studies have revealed the identity of its heterogeneous protein/peptide composition and their abundance on the surface of QD-HC (section 3.3.2). The four major proteins that constitute the hard protein corona by relative abundance were apolipoprotein B-100, serum albumin, complement C3 and apolipoprotein A-1. It was estimated that the outermost layer of the hard protein corona may primarily consists of apolipoprotein B-100 proteins due to the reasons stated prior, thereby making it the candidate protein that interfaces with the plasma membrane of the cells. Provided that the native epitope of this protein was structurally intact and well exposed to be properly recognized by the cell surface receptors (Mahmoudi et al., 2013b), internalization of QD-HC will be promoted because apolipoprotein B-100 naturally functions as a transport protein that traffics lipid and cholesterol molecules across the cells (Schottler et al., 2016). Studies have reported that nanoparticles with apolipoprotein enriched protein coronas and nanoparticles that were surface functionalized with purified apolipoproteins were internalized at higher rate by the cells compared to the controls (Kreuter, 2004; Ogawara et al., 2004). The mechanistic details of apolipoprotein B-100’s involvement in promoting the receptor mediated endocytosis of QD-HC was not clear and no further attempts were made to clarify the process by experimentation as it was beyond the scope of the present study.
**Figure 5.10:** Internalization of QDs in (A) IMR90 cells and (B) CCD841CoN cells cultured with and without 15% FBS. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
5.3.5 Localization of nanoparticles within cell

Fluorescence microscopy analysis have revealed that, at later phases of internalization the QDs were trafficked to lysosomes of both young and senescent cells. QDs were predominantly colocalized in this subcellular organelle and there was no evidence of its mass localization in any other region within the cellular compartments (Figures 5.11 & 5.12). The senescent cells have higher volume of lysosomes compared to the young cells based on the lysosomal staining pattern as depicted in Figure 5.13, which was in agreement with other studies comparing the lysosomal content of young and senescent cells (Kurz et al., 2000b). These studies have also reported that the mean size of individual lysosomes were larger in senescent cells due to the presence of lipofuscin and other oxidized materials within it (Brunk and Terman, 2002).

The presence of lipofuscin pigments in senescent cells were revealed by live fluorescence microscopy analysis as depicted in Figure 5.14. Lipofuscins are natural fluorophores that autofluoresces when excited with ultraviolet light (Mochizuki et al., 1995). The monochromatic image shows a nucleus being orbited by a dense cloud of clustered lipofuscin granules within the lysosomal compartment of viable senescent cell. The nucleus in the image were stained with nuclear specific Hoechst fluorophore and the resulting emission from the organelle exposed its well defined structure, which implied that the proximal illumination in the image originated from the autofluorescing lipofuscin pigments. Figure 5.14C shows the juxtaposition of QD nanoparticles within the same region of lipofuscin, thus confirming its presence in lysosomal compartments of senescent cells. Parallel comparison of the images obtained from nuclear staining of young and
senescent cells have indicate the absence of lipofuscin pigments in the former (Figure 5.15).

The contention that larger number of QDs will occupy the lysosomal compartment of senescent cells compared to young cells due to the sheer size and volume of lysosomes in the former was validated by the colocalization study. The acidic lysosomal compartments were fully encompassed by the QDs within 1.5 hours of incubation in young cells and 1.5 - 2.5 hours in senescent cells. The difference in the length of time it took for the QDs to fully encompass the lysosomal compartment of young and senescent cells can be attributed to the size difference between the cells. The mean size of senescent cells was much larger than young cells and this expanse was manifested as laterally stretched out cell membrane region covering large surface areas in the culture vessel.

QDs that were endocytosed at regions of the senescent cells that were further away from lysosomes were trafficked to it by the endosomes and the interim trafficking process may take longer as opposed to the QDs that were internalized in the region closer to the lysosomes within the same cell. Even though the senescent cells have higher volume of lysosomes compared to young cells, yet they were not distributed evenly throughout the cytoplasm and were mostly scattered around the perinuclear region similar to the young cells. Another factor that could have contributed to the delay in QDs’ accumulation within the lysosomes of senescent cells were its amount; higher amount of QDs were required to fully encompass the senescent cells’ lysosomes due to its larger size and volume.
**Figure 5.11:** Fluorescence micrographs of QDs colocalization in lysosomes of young IMR90 and CCD841CoN cells. Experiments were carried out at 37°C for 2h. Lysosomes were stained with LysoTracker Blue fluorescent dye (blue) and QD-PEG/QD-HC nanoparticles were marked in red. Bar = 50µm.
Figure 5.12: Fluorescence micrographs of QDs colocalization in lysosomes of senescent IMR90 and CCD841CoN cells. Experiments were carried out at 37°C for 2h. Lysosomes were stained with LysoTracker Blue fluorescent dye (blue) and QD-PEG/QD-HC nanoparticles were marked in red. Bar = 50µm.
Figure 5.13: Fluorescence micrographs of lysosomes in young and senescent (A) IMR90 cells and (B) CCD841CoN cells. The cells were stained with LysoTracker Blue fluorescent dye. Bar = 50µm.
Continued…….
Figure 5.14: (A) Monochromatic fluorescent micrograph depicting Hoechst 33342 stained nucleus of IMR90 senescent cell. The nucleus was marked as (N) and the arrows depict lipofuscin pigments. (B) Monochromatic fluorescent micrograph portraying localization of quantum dots (red circle) within the senescent cells. (C) Merged fluorescent image of stained nucleus (blue) and quantum dots (red) in pseudocolours, marked as N and QD, respectively. Bar = 50µm.
Figure 5.15: Monochromatic fluorescent micrograph depicting Hoechst 33342 stained nucleus of (A) young and (B) senescent IMR90 cells. Bar = 50µm.
5.4 Conclusion

Based on the results, it was shown that the predominant route of entry into the cells for all the QDs were through endocytic pathway. Upon further probing, it was established that all the cells utilized clathrin dependent endocytosis as the primary mechanism to traffic in the QDs. The internalization of QDs by the cells were largely dependent on the QDs’ sediment size and surface characteristics; whilst the ability of cells to internalize the QDs were cell type dependent. Transformation of young cells into senescent phenotype had altered its physical and biochemical features, which consequently had affected its propensity to internalize the QDs. The endocytosis efficacy in the senescent cells were clearly compromised, resulting in slower internalization of the QDs.

Nanoparticle agglomeration and gravitational sedimentation which constitutes the tangible size of QDs in the culture medium played a key role in its endocytosis with smaller ones being internalized more rapidly than the larger ones by all the cells. The affairs of nanoparticle internalization were also influenced by its surface properties, whereby hard protein coated QDs (QD-HC) were internalized at a higher rate compared to PEGylated QDs (QD-PEG) by all the cells. It was also revealed that, specific protein(s) within the hard protein corona layer may have aided the internalization of the QDs rather than its surface charge.

Another important aspect of cellular dynamism with regards to cell-nanoparticle interaction was the exocytosis process. The exocytosis of QDs were significantly retarded
in senescent cells relative to the young cells. Moreover, the departure of QDs from senescent cells were more exotic compared to the young version of both cells irrespective of the QD type. Within the context of senescent cells, smaller QDs were released more swiftly compared to the larger ones.

After internalization, all the QDs were trafficked into the lysosomal compartment of young and senescent cells. However, the QD’s amount and accumulation rate within this organelle differs between the young and senescent cells, primarily due to the structural changes associated with the senescence phenotype.
CHAPTER SIX: IN VITRO CYTOTOXICITY OF NANOPARTICLES

6.1 Introduction

*In vitro* cytotoxicity assays are useful tools to assess the toxic potential of chemical substances or nanomaterials. Enhanced sensitivity and reproducibility of measurement results are some of the key features of the *in vitro* cytotoxicity assay systems. Utilizing the multitude of available cytotoxic assays, the exact nature and degree of damage can be determined in a quantifiable manner. Concurrently, the safe dosage of the chemical substances or nanomaterials can be determined by performing acute and chronic cytotoxic studies. These data can then be extrapolated and utilized in *in vivo* animal studies or human clinical trials. The most important aspect of the current study was to determine the deleterious effects of the quantum dots on senescent and non-senescent cells. To determine the cytotoxic potential of the quantum dots, different endpoint cytotoxicity assays were performed to quantify the cell viability.
6.2 Materials and methods

6.2.1 WST-1 cell viability assay

10% (v/v) WST-1 were prepared in DMEM without phenol red and serum. After the incubation period, the medium in wells of treated cells were removed and the cells were rinsed using sterile PBS three times. After the washing steps, prepared WST-1 solution were added in the wells and incubated for 2-4 hours at 37°C in CO$_2$ incubator. Finally, the absorbance of samples was measured at 450 nm using spectrophotometer and the absorbance directly correlates to the number of viable cells. WST-1 calibration curve for all the cells assayed in the experiment is shown in Figure 6.1. The assays were performed on 10$^4$ cells/well in six replicates and three independent experiments. Acetaminophen (25 mM) in DMEM medium was used positive control in this experiment and nanoparticle interference with the WST-1 assay were tested in DMEM medium without cells.

6.2.2 Neutral red retention (NRR) assay

0.33% Neutral Red Solution were added in an amount equal to 10% (v/v) of DMEM without phenol red and serum. After the incubation period, the medium in wells of treated cells were removed and the cells were rinsed using sterile PBS three times. After the washing steps, prepared Neutral Red solution were added in the wells and incubated for 2-4 hours at 37°C in CO$_2$ incubator. At the end of the incubation period, the medium was carefully removed and the cells were quickly rinsed with Neutral Red Assay Fixative. The fixative solution was removed and the incorporated dye is then solubilized in a
volume of Neutral Red Assay Solubilisation Solution equal to the original volume of culture medium. The cultures are allowed to stand for 10 minutes at room temperature and stirred gently in a gyratory shaker to enhance mixing of the solubilized dye. Finally, the absorbance of samples was measured at 540 nm using spectrophotometer and the absorbance directly correlates to the number of viable cells. NRR calibration curve for all the cells assayed in the experiment is shown in Figure 6.2. The assays were performed on $10^4$ cells/well in six replicates and three independent experiments. Triton X-100 (1%) in DMEM medium was used positive control in this experiment and nanoparticle interference with the WST-1 assay were tested in DMEM medium without cells.

**6.2.3 Lactate dehydrogenase (LDH) release assay**

LDH release assay was performed using Pierce LDH cytotoxicity assay kit as recommended by the manufacturer. The absorbance of samples was measured at 490 nm using spectrophotometer and the absorbance directly correlates to the number of lysed cells. LDH calibration curve for all the cells assayed in the experiment is shown in Figure 6.3. The assays were performed on $10^4$ cells/well in six replicates and three independent experiments. Triton X-100 (1%) in DMEM medium was used as positive/maximum LDH activity control in this experiment and nanoparticle interference with the LDH release assay were tested in DMEM medium without cells.
Figure 6.1: Cell titration curve for WST-1 assay of (A) IMR90, (B) CCD841CoN, and (C) primary fibroblast cells.
Figure 6.2: Cell titration curve for NRR assay of (A) IMR90 and (B) CCD841CoN cells.
Figure 6.3: Cell titration curve for LDH assay of (A) IMR90 and (B) CCD841CoN cells.
6.2.4 Lysosomal alkalization

Lysosomal alkalization of the senescent cells was performed by pre-treating the cultures with NH$_4$Cl (30 mM) prepared in DMEM for 4 h prior to QD nanoparticle incubation. After incubation, the solutions were removed and the cells were washed 2X with PBS for 2 minutes each. QD nanoparticles prepared in cell culture medium were added in the wells and incubated for 24 hours at 37°C in CO$_2$ incubator. The solutions were then removed and cells were washed three times with PBS before proceeding with WST-1 cell viability assay.

6.2.5 Volume of acidic compartment (VAC) measurement

LysoTracker blue (120 nM) were prepared in DMEM without phenol red and serum. After the incubation period, the medium in wells of young and senescent cells were removed and the cells were rinsed using sterile PBS three times. After the washing steps, prepared LysoTracker blue solution were added in the wells and incubated for 1 h at 37°C in CO$_2$ incubator. After incubation, the medium in wells were removed and cells were rinsed using sterile PBS three times. Cells were then trypsinized, pelleted and resuspended in 1 ml of PBS and measured using fluorescence spectrophotometer at excitation/emission wavelength of 370/422 nm. Mean value of fluorescence intensity was assigned as the relative value of VAC.
6.2.6 Cadmium toxicity

Molecular weight of QD705 is $1.5 \times 10^6$ g and the abundance of Cd in QD705 is $56.16 \pm 8.54\%$. 200µL of 2 µM concentration QD705 contains 400 pmol of quantum dots with molecular weight of 600 µg and the amount of Cd in 400 pmol of QD705 is 336.9 µg. Highest concentration of Qtracker 705 used in the cytotoxicity experiment was 45 µM which contains 90 pmol of quantum dot and therefore 75.8 µg of Cd. 375 µM CdCl₂ was used in the experiment that contains equivalent amount of Cd in 45 µM QD705. IMR90 and CCD841CoN cells were incubated in 375 µM CdCl₂ dispersed in cell culture medium for 24 h at 37°C in CO₂ incubator. The solutions were then removed and cells were washed three times with PBS before proceeding with WST-1 cell viability assay.

6.2.7 Derivative toxicity due to nanoparticle leaching and disintegration

QD nanoparticles were incubated in ALF solution to simulate lysosomal pH at 4.5–5.0 and prepared as described by Stopford et al. (2003). The solution was centrifuged to pellet the QDs and supernatant was collected and pH was adjusted to 7.2 before dispersing in cell culture medium. IMR90 and CCD841CoN cells were incubated in the supernatant for 24 h at 37°C in CO₂ incubator. The solutions were then removed and cells were washed three times with PBS before proceeding with WST-1 cell viability assay.
6.2.8 Autophagy and apoptosis inhibitor treatment of cells

To determine the type of cell death caused by cadmium chloride and the QD supernatants on the senescent cells, the cultures were pre-treated with apoptosis inhibitor ZVAD-FMK and autophagy inhibitor 3-MA. IMR90 and CCD841CoN senescent cells were pre-treated with 25-30 μM ZVAD-FMK for 1h or 5-7 μM 3-MA for 24h before incubation with cadmium chloride and the QD supernatants. After incubation for 24 hours at 37°C in CO₂ incubator, the solutions were then removed and cells were washed three times with PBS before proceeding with WST-1 cell viability assay.

6.2.9 Statistical analyses

Statistical analysis was performed based on one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer post hoc test of significance by using Prism® 5.0 software (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered to be statistically significant. Cytotoxicity results are expressed as mean ± SD of one representative experiment performed in 6 replicates, and the experiments were repeated three times. GraphPad Prism® 5.0 software (GraphPad, San Diego, CA, USA) was used to calculate the concentrations associated with 50% viability (IC50 values) with 95% confidence limits using a Hill function nonlinear regression analysis.
6.3 Results and Discussion

6.3.1 Acute cytotoxic effects of nanoparticles

Acute cytotoxicity assays were performed on young and senescent cells to determine the toxic potential of QD-PEG and QD-HC during 24 hours incubation period with WST-1 metabolic assay. To gain deeper insight into the effects of these QDs, young cells were subdivided into contact inhibited and proliferating cells; whilst the senescent cells were subdivided into replicative senescence, oxidative stress induced and genotoxic stress induced cells. To determine the correlation between induced cellular senescence and chronological lifespan of the cells, cytotoxicity assays were also performed on primary fibroblast cells of different age groups: AG04450 fetal human lung fibroblasts (12 weeks), AG02603 adult human lung fibroblasts (35 years) and AG02262 adult human lung fibroblasts (61 years).

Based on the results, no cytotoxic effect was observed on the young cells as measured by the percentage of viable cells against nanoparticle concentrations (Figure 6.4B & C). Similarly, no cytotoxic effect was observed on all the primary fibroblast cells of different age groups (Figure 6.4A). On the contrary, QD-PEG and QD-HC had exerted toxic effects on all the different senescent cell types in a dose dependent manner (Figure 6.5).

More appropriately by using IC50 as an index to gauge the toxic potential of the nanoparticles, QD-PEG was determined to be more toxic to the senescent cells compared
to QD-HC within the concentration range tested. IC50 was the concentration of QDs that were needed to inhibit the metabolic activity of half the cell population and higher IC50 values indicate lower cytotoxic potential and vice versa. IC50 values of QD-PEG were in the range of 18-20 nM and IC50 values of QD-HC were in the range 30-40 nM, indicating that the former was approximately twice as toxic compared to the latter on CCD841CoN and IMR90 senescent cells. In fact, at low concentrations of 15nM, QD-HC treatment did not induce significant toxic effect on the senescent cells compared to the controls; whilst QD-PEG treatment had induced 42% cell death on senescent IMR90 and 51% cell death on senescent CCD841CoN compared to the controls at 24h hour incubation (Figure 6.6).

There was no significant difference in the IC50 values within the senescent cell types as a result of QD-PEG and QD-HC treatment, indicating there was a common mode of inhibition exerted upon the senescent cells regardless of the ageing methods used. If the enabling factor for the inhibition was a property of the senescent phenotype that was imbued during the ageing transformation process, then it could adequately explain the difference in the cytotoxicity response that was exhibited by the young and senescent cells.

Adding credence to this theory was the fact, primary fibroblast cells of different age groups did not show any variation in their cytotoxic response towards the QDs. The supposition was that, AG04450 and young cells would have parallel toxic response, AG02603 would have intermediary toxic response between young and senescent cells, and AG02262 and senescent cells would have parallel toxic response based on the
chronological age of the cells. Except for AG04450, the two other primary fibroblast cells did not adhere to the aforementioned supposition and interestingly both of these cells did not show any characteristics of the senescence phenotype based on the cell ageing biomarker analysis (section 4.3.4). The current data have suggested a strong correlation between the senescence phenotype and its deleterious cytotoxic response towards the QDs without linking it to a particular or an ensemble of senescence traits for its frailty.

Dilution of QDs in the young cells via cell division can mitigate its cytotoxic effects, as toxicity was dose dependent based on the experimental data. Even though cell division of the IMR90 and CCD841CoN cells occur at approximately 24 hours, which coincides with the current cytotoxicity experimental setup, the cells in the culture were not synchronized to undergo symmetrical cell cycle phase. Hence, cell division occurs stochastically within the cell population and there was a distinct possibility that dilution of the QDs can ensue (Kim et al., 2011).

To circumvent this problem, contact inhibited young cells were also used for this experiment, which essentially has similar metabolic activity as the proliferating cells but does not undergo cell division (Marthandan et al., 2014). Therefore, the argument that nanoparticle dilution via cell division was a mechanism by which the young cells were able to mitigate the cytotoxic potential of the QDs was invalidated.
Table 6.1: Cytotoxic effect of nanoparticles on young, senescent and primary cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>QD-PEG (nM)</th>
<th>QD-HC (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;100&lt;/sub&gt;</td>
</tr>
<tr>
<td>IMR90 - Replicating</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>IMR90 - Contact inhibited</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CCD841CoN - Replicating</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CCD841CoN - Contact inhibited</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>IMR90 - Replicative senescence</td>
<td>13.88 ± 0.44</td>
<td>27.50 ± 0.65</td>
</tr>
<tr>
<td>IMR90 - Hydrogen peroxide treated</td>
<td>13.10 ± 0.85</td>
<td>25.35 ± 0.72</td>
</tr>
<tr>
<td>IMR90 - Doxorubicin treated</td>
<td>14.05 ± 0.63</td>
<td>26.45 ± 0.25</td>
</tr>
<tr>
<td>CCD841CoN - Replicative senescence</td>
<td>15.74 ± 0.65</td>
<td>28.50 ± 0.35</td>
</tr>
<tr>
<td>CCD841CoN - Hydrogen peroxide treated</td>
<td>15.32 ± 0.77</td>
<td>30.77 ± 0.58</td>
</tr>
<tr>
<td>CCD841CoN - Doxorubicin treated</td>
<td>16.01 ± 0.95</td>
<td>29.33 ± 0.75</td>
</tr>
<tr>
<td>AG04450 – 12 weeks</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>AG02603 – 35 years</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>AG02262 – 61 years</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SD. n=6 for each group.

IC<sub>50</sub>: Concentration of nanoparticles inhibiting 50% of cell population.

IC<sub>100</sub>: Concentration of nanoparticles inhibiting 100% of cell population.

N.D.: Not determined at the concentration range tested (0-50 nM).
Continued...
Figure 6.4: Acute cytotoxicity of QD nanoparticles on (A) young IMR90 cells, (B) young CCD841CoN cells and (C) primary fibroblast cells of different age groups. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with different concentrations of QD-PEG and QD-HC nanoparticles. The highest concentration of nanoparticles tested for each experiment was 50 nM. Data are represented as mean ± SD. n=6 for each group.
Figure 6.5: Acute cytotoxicity of QD nanoparticles on senescent (A) IMR90 cells and (B) CCD841CoN cells. (C) IC\textsubscript{50} values of nanoparticles on senescent IMR90 and CD841CoN cells. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with different concentrations of QD-PEG and QD-HC nanoparticles. The highest concentration of nanoparticles tested for each experiment was 50 nM. IC\textsubscript{50} values represent concentration of nanoparticles required to inhibit 50\% of the total cell population. Control: young IMR90 and CCD841CoN cells, H\textsubscript{2}O\textsubscript{2}: hydrogen peroxide, DOX: doxorubicin. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Figure 6.6: Comparison of QD nanoparticles toxicity on senescent (A) IMR90 and (B) CCD841CoN cells. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with specific concentrations of QD-PEG and QD-HC nanoparticles. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
6.3.2 Effect of protein corona on nanoparticle toxicity

Flow chart on Figure 6.7 depicts all the possible outcomes of the cytotoxicity results and the influence of protein corona within this scheme. The chart was a summary of binding conditions that must occur for comparisons to be made within and between the cell types to determine the effect of protein corona. If QD-PEG acts as an agonist to induce some response in the cells and QD-HC acts as an antagonist to this response or vice versa, then protein corona effect was manifested. Since the response in this experiment was measured by quantifying the cell viability, there should always be a disparity between the cytotoxic response invoked by QD-PEG and QD-HC ($T_A > T_B$ or $T_B > T_A$) on young or senescent cells or both for the protein corona effect to be legitimate.

Cytotoxic response of the cells was directly linked to the cytotoxic potential of the QDs and the two parameters that were selected to differentiate the cytotoxic potential between the QDs were: (1) QD with the highest number of cell mortality at the end of 24 hour incubation period, and (2) in the event that both QDs were able to reduce the cell population to zero at the end of 24 hour incubation period, the QD that takes the shortest time to induce 50% cell mortality. The highlighted (yellow) parts on the flow chart indicates the cytotoxicity results obtained from the experiments and the trail of protein corona involvement.

Protein corona had profound effect on the toxicity of QDs on senescent cells of both types. QD-HC was less cytotoxic than QD-PEG and the only distinguishing feature
between these nanoparticles was the presence of hard protein corona on the former. Therefore, it was reasonable to conclude that the presence of this protein layer had attenuated QD’s toxicity after eliminating all the other influencing elements in the current experimental setup. The mitigation of nanoparticle’s cytotoxic effects by the presence of protein corona had been widely reported (Hu et al., 2011a; Shi et al., 2012; Docter et al., 2014).

On the contrary, hard protein corona had no net effect on the toxicity of QDs in young cells of both cell types. Based on corollary interpretation from toxicity data of senescent cells, it could be contended that the effect of protein corona on young cells were not discernible rather than nonexistent. This deduction was based on the grounds that protein corona mitigates the negative effects of the QDs. Since, QDs did not induce deleterious effects on young cells, the causative factor had been nullified and the detoxifying potential of protein corona was inconsequential in this context. The results from the study did not present ambiguity with regards to the effect of protein corona; however, it remains to be known on how protein corona coatings on QD had attenuated its toxicity at this stage of the experiment.
Figure 6.7: Flow chart summarizing the possible permutations of the cytotoxicity results and the effect of protein corona within this scheme.
6.3.3 Effect of cell size on nanoparticle toxicity

The data suggests that the size of the cells can also influence its cytotoxic response towards the QDs. The sizes of senescent cells were significantly larger (P < 0.001) than young cells for both cell types based on fluorescence microscopy analysis (Figure 5.3). However, the influence of other physical and biochemical features that were distinct to the young and senescent phenotype must be taken into consideration prior to making quantitative assessment on the interrelationship between cell size and QD toxicity. Hence, the current data on the correlation between cell size and QD toxicity was obtained by exclusively evaluating senescent cells in different spatial configurations, which ensured that the assessment was reliably valid as all the other experimental variables were normalized. To be more precise, exposed cell surface region was a better description than cell size with regards to the current evaluation. The emergence of senescent cell’s altered spatial configuration or layout on the culture flask transpired as a consequence of in vitro cell manipulation.

This phenomenon was very obvious in replicative senescence, as the experimental procedure to induce cellular senescence required serial passaging of the cells, which involves repeated cycles of detachment and reattachment of cells onto the surface of culture flasks. As the cell passage number was increased, the cell’s size also increased gradually during its transition into senescence state (section 4.3.1.1). The size increase in the senescent cells occurred via cytoskeleton reorganization that was primarily attributed to the alterations in the amount and type of vimentin proteins produced by the ageing cells (Nishio and Inoue, 2005). When the senescent cells were re-cultured, these elongated and
pliable cells were muddled during the gravitational settling process as they descend towards the bottom of the culture vessel.

As the cells reattach onto the surface of culture flask, they were found to be folded over with only parts of the cells being exposed compared to their full surface area. Hence, depending on the cell culture condition, the topological layout was heterogeneous but always less exposed compared to the pre-passaged senescent cells (Figure 6.8). In premature induced senescent cells, either through hydrogen peroxide or doxorubicin exposure, cell fold over does not occur because the cells were induced in situ and the enlargement of cell and its spreading/flattening occurred locally in the culture flask.

The temporal analysis data have shown that the population of senescent cells with larger size or with more exposed surface area were reduced at faster rate compared to the folded senescent cells of both cell types as a consequence of QD toxicity (Figures 6.9 & 6.10). Therefore, QD’s toxicity was positively correlated to the cell’s surface area; thus establishing a causal relationship between the observed delays in cell mortality and the magnitude of cell’s exposed surface area for the folded and normal senescent cells. It was hypothesized that, in the folded senescent cells, its less exposed surface area had reduced its probability of interacting and internalizing the QDs relative to the normal senescent cells. Since the QD’s toxicity was concentration dependent, higher accumulation of QDs via endocytosis will induce toxicity on the cells at a faster rate and vice versa.
Temporal analysis on QD’s toxicity had indicated that the delay in cell mortality between the normal and folded senescent cell were significantly higher (P < 0.001) in IMR90 cells (Figure 6.9) compared to the CCD841CoN cells (Figure 6.10). Once again, this observation can be explained on the basis of earlier established causal relationship between the elapsed time in cell mortality and the magnitude of cell’s exposed surface area. The mean sizes of normal and folded senescent IMR90 cells were 54 ± 4.5µm and 20 ± 2µm, respectively and the percentage of size decrease in the folded cells were 62.96%. The mean sizes of normal and folded senescent CCD841CoN cells were 18 ± 1.5µm and 8.5 ± 0.5µm, respectively and the percentage of size decrease in the folded cells were 52.77%.

The percentage of size difference between the senescent IMR90 cells were higher than the percentage of size difference between the senescent CCD841CoN cells, which means the cytotoxic response will be more drastically reflected between the normal and folded state in the former by greater delay in cell population reductions compared to the latter. Based on this data, it was conjectured that the cell’s size could be one of the factors as to why the smaller sized young cells were not lethally affected by the QD’s toxicity compared to the larger sized senescent cells.
Figure 6.8: Fluorescence micrographs of (A) senescent IMR90 cells, (B) folded senescent IMR90 cells, (C) senescent CCD841CoN cells, and (D) folded senescent CCD841CoN cells. Folded senescent cells have less exposed surface area compared to the normal senescent cells. The cells were stained with CMFDA CellTracker fluorescent dye. Bar = 50µm.
Figure 6.9: (A) Temporal analysis and (B) quantification of QD nanoparticle toxicity on normal and folded senescent IMR90 cells. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with specific concentrations of QD-PEG and QD-HC nanoparticles. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Figure 6.10: (A) Temporal analysis and (B) quantification of QD nanoparticle toxicity on normal and folded senescent CCD841CoN cells. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with specific concentrations of QD-PEG and QD-HC nanoparticles. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
6.3.4 Modality and mechanism of senescent cell death induced by nanoparticles

The major organelles in cells are intricately linked for its proper functioning and disruption to this system will affect its viability or proliferation. For instance, damage to a particular organelle will disrupt the activity of another organelle located downstream or upstream of a cellular pathway leading to cellular dysfunction. Therefore, it was important to establish the sequence of events leading to cell death based on temporal analysis in the current study. Temporal analysis was performed to identify the primary target of the QDs and its secondary effects on the other organelles. Three different end point assays were employed to assess the cellular damage or toxicity on senescent cells. Neutral Red Retention (NRR) assay was used to detect lysosomal damages in the cells, WST-1 assay was used to detect mitochondrial damages in the cells and LDH (Lactate Dehydrogenase) assay was used to detect plasma membrane integrity of the cells. The assays were performed with fixed QD concentrations (IC50) obtained from earlier cell viability results and the readings were taken at fixed time intervals within 24 hours.

Based on the results, the sequence of events with regards to detectable cellular damage began with lysosome, followed by mitochondria and finally plasma membrane in both the senescent cell types (Figures 6.11 & 6.12). Evidently, the localized perturbation exerted by the QDs within the lysosomal compartments of senescent cells resulted in a cascade of events that led to its death by necrosis.
Another common cytotoxicity pattern observed in the present study was the delay in the cell death induction by 45nM QD-HC (Figures 6.11B & 6.12B) compared to 30nM QD-PEG (Figures 6.11A & 6.12A). In both senescent cell types, complete cell death occurred within 16 hours of treatment with 30nM QD-PEG; whilst, it required 24 hours for the 45nM QD-HC to induce complete cell death. It was postulated that the presence of hard corona layer surrounding QD-HC had delayed its degradation by the hydrolytic enzymes inside the lysosomes; thus, delaying the leaching of Cd\textsuperscript{2+} from QD-HC and inducing its deleterious effects on the senescent cells (Cho et al., 2012) (section 6.3.2).

The results have also suggested that the QD’s toxic effects on the senescent cells were cell type dependent. Characteristic difference being the rapid succession of mitochondrial dysfunction soon after lysosomal impairment on senescent CCD841CoN cells relative to IMR90 cells. Mitochondrial-lysosomal axis theory of ageing explains the continuous deterioration of these two organelles are interrelated and occurs in an amplifying feedback loop which accelerates the ageing of the cells (Terman et al., 1999). It was presumed that mitochondrial-lysosomal pathway was tightly coupled in the senescent CCD841CoN cells compared to IMR90 cells.
Figure 6.11: Temporal analysis of QD nanoparticles toxicity on senescent IMR90 cells with (A) 30 nM QD-PEG and (B) 45 nM QD-HC. Toxicity measurement was performed using WST-1 colorimetric assay, neutral red retention (NRR) assay and lactate dehydrogenase (LDH) assay after 24h incubation. H$_2$O$_2$: hydrogen peroxide, DOX: doxorubicin. Data are represented as mean ± SD. n=6 for each group.
Figure 6.12: Temporal analysis of QD nanoparticles toxicity on senescent CCD841CoN cells with (A) 30 nM QD-PEG and (B) 45 nM QD-HC. Toxicity measurement was performed using WST-1 colorimetric assay, neutral red retention (NRR) assay and lactate dehydrogenase (LDH) assay after 24h incubation. H₂O₂: hydrogen peroxide, DOX: doxorubicin. Data are represented as mean ± SD. n=6 for each group.
It was hypothesized that lysosomal membrane permeabilization induced necrosis was the primary mode of cell death observed in these senescent cells. Cadmium ions (Cd\(^{2+}\)) were considered to be the source that actuated the toxic effects in these cells as it was the major constituent of the QDs and also a widely recognized cytotoxic agent. The toxic effects of Cd\(^{2+}\) on various types of cells had been extensively reported (Galan et al., 2001; Ishido et al., 2002). Cadmium molecules at the core of the QDs were covalently bonded with other molecules such as selenium or telluride and surrounded by a layer of zinc sulfide to prevent the QD’s leaching in physiological solutions; thus, the intact QDs will be nontoxic to the cells (Dabbousi et al., 1997). However, the integrity of the QDs can be compromised under non-physiological conditions such as in highly oxidative or acidic environments (Soenen et al., 2012).

As discussed previously, the QDs were internalized by the cells via endocytosis and transported to the lysosomes in endosomal compartments (section 5.3.5). After the endosome fuses with the lysosome, the QDs were degraded in this highly oxidative environment by hydrolytic enzymes resulting in the release of its Cd\(^{2+}\) into the lumen of lysosomes (Soenen et al., 2012). The acidification of lysosomal lumen by the release of Cd\(^{2+}\) will create instability in this compartment; thus, increasing the permeability of its membrane (Figures 6.13A & 6.14A). As a consequence of lysosomal membrane permeabilization, the Cd\(^{2+}\) will leak into the cytosol and interfere with the functioning of other cellular organelles such as mitochondria. Fate of the cells will depend on the rate of Cd\(^{2+}\) leakage into the cytosol and the cells’ ability to chelate these ions. Continuous disruption to the metabolic flux of the cell will trigger programmatic cell death such as
apoptosis or autophagy (Wang et al., 2008). In the case of abrupt flooding of Cd$^{2+}$ into the cytosol due to lysosomal rupture, the effects on the cells will be even more detrimental as hydrolytic enzymes were also released into the cytosol causing massive cellular damage and cell death by necrosis. Further analysis using fluorescence microscopy have confirmed this to be the case (Figures 6.13B & 6.14B).

To test the hypothesis that lysosomal membrane permeabilization induced necrosis was indeed the cause of cell death, senescent cells were pre-treated with ammonium chloride for lysosomal alkalization before incubation with the QDs. There was significant reduction (P < 0.001) in the toxicity levels for CCD841CoN and IMR90 senescent cells compared to untreated control (Figures 6.15 & 6.16). Reduction in the cytotoxicity based on QD’s concentration was as follows: 30nM QD-PEG (75%) and 45nM QD-HC (80%) in senescent IMR90 cells (Figure 6.15B) and 30nM QD-PEG (73%) and 45nM QD-HC (80%) in senescent CCD841CoN cells (Figure 6.16B). By preventing the acidification of lysosomes, the breakdown of QDs and the release of its byproducts such as Cd$^{2+}$ were decelerated, which otherwise would have intensified the destabilization of lysosomal lumen leading to the increased permeabilization of its membranes and eventual collapse of the lysosomal structure. The destruction of lysosomes was accompanied by the release of its hydrolytic enzymes into cytoplasm, which subsequently triggered the cells to expire by necrosis as revealed by the LDH results.
Continued……
Figure 6.13: (A) Fluorescence micrographs of lysosomal membrane permeabilization in senescent IMR90 cell due to QD toxicity. (B) Fluorescence micrographs of QD toxicity on senescent IMR90 cell. Micrographs are ordered from (a-i) to correspond with the progression of incubation time. Release of QD nanoparticles from cell due to plasma membrane leakage indicates necrotic cell death. Experiments were carried out at 37°C for 24h. Cells were stained with CMFDA CellTracker fluorescent dye (green), lysosomes were stained with LysoTracker Blue fluorescent dye (blue) and QD-PEG nanoparticles were marked in red. Bar = 50µm.
Continued…….
**Figure 6.14:** (A) Fluorescence micrographs of lysosomal membrane permeabilization in senescent CCD841CoN cells due to QD toxicity. (B) Fluorescence micrographs of QD toxicity on senescent CCD841CoN cells. Micrographs are ordered from (a-i) to correspond with the progression of incubation time. Release of QD nanoparticles from cell due to plasma membrane leakage indicates necrotic cell death. Experiments were carried out at 37°C for 24h. Cells were stained with CMFDA CellTracker fluorescent dye (green), lysosomes were stained with LysoTracker Blue fluorescent dye (blue) and QD-PEG nanoparticles were marked in red. Bar = 50µm.
Figure 6.15: (A) Temporal analysis and (B) quantification of QD nanoparticle toxicity on normal and ammonium chloride pre-treated senescent IMR90 cells. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with specific concentrations of QD-PEG and QD-HC nanoparticles. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Figure 6.16: (A) Temporal analysis and (B) quantification of QD nanoparticle toxicity on normal and ammonium chloride pre-treated senescent CCD841CoN cells. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with specific concentrations of QD-PEG and QD-HC nanoparticles. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
To resolve as to why this only occurred in the senescent cells and not in the young cells, the structure and functionality of lysosomes in senescent cell should be examined because this organelle was the pivotal point of toxic manifestation and its containment by alkalization had resulted in reduced cell mortality. Volume and size increase of lysosomes in senescent cells relative to the young cells was evidenced by micrographs obtained from lysotracker staining of acidic compartments (Figure 5.13). The size and volume increase of lysosomes in senescent cells were mainly attributed to the presence of lipofuscin, which is a cellular marker for ageing (Brunk and Terman, 2002). Lipofuscin are non-degradable oxidized proteins that accumulate in cell’s lysosomal compartments and in senescent cells, a large majority of its lysosomes are loaded with this material. Given that lipofuscin are non-degradable matter, they are not dismantled or exocytosed from the cells but are retained in lysosomes. In proliferating cells, the amount of lipofuscin is diluted by cell division, however in post mitotic cells this is not an option. In the absence of cellular mechanisms to effectively eliminate or expel lipofuscin from post-mitotic cells, its content will increase over time as a derivative byproduct of steady state autophagic activity to maintain cellular homeostasis. The enlargement of lysosomes occurs due the expansion of lysosomal lumen to accommodate increasing amounts of lipofuscin that were translocated from cytosol by autophagosomes.

Some studies have concluded that size increase in lysosomal compartment was positively correlated to the accumulation of lipofuscin in senescent cells (Kurz et al., 2008). In light of the fact that lysosomes rich in lipofuscin pigments are below par at degrading waste materials, cellular biogenesis of this organelle will surge to compensate
for the waning lysosomal activity, which explains the increase in total lysosomal volume of senescent cells (Kurz et al., 2000b). Volume of intracellular acidic compartments per cell (VAC) measurement was used to quantify the lysosomal content in the young and senescent cells. The data on VAC measurements have shown an increase of approximately 400% in senescent IMR90 cells and 385% in senescent CCD841CoN cells compared to the young cells of each type (Figure 6.17). In an extensive study carried out by Ono et al. (Ono et al., 2003) on the mode of cell death relating to the size of lysosomes exemplified by VAC, they have strongly linked VAC increase to plasma membrane disruption leading to cell death via necrosis. The current data corroborates their findings in linking VAC increase to necrotic cell death.

Based on the results, further introspection into QDs’ colocalization within lipofuscin loaded lysosomes was vital to understand the devastating consequences on the viability of senescent cells. Chiefly the physical and biochemical features of lipofuscin loaded lysosomes that makes it susceptible to insults by QDs must be examined. By default, the structural integrity of large lysosomes were compromised because the size expanse reduces its overall tensile strength and increases its fragility (Gomez-Sintes et al., 2016). In addition, modification in lipid composition of lysosomal membranes in senescent cells, especially an increase in its lysophosphatidylcholine content can reduce the integrity of lysosomal membrane by increasing its permeability (Gomez-Sintes et al., 2016). Further assault on these defective lysosomes will accelerate its rupture and subsequently its total collapse; thus, releasing the entirety of its content into the cell’s cytosol.
**Figure 6.17:** Lysosomal content of young and senescent IMR90 and CCD841CoN cells. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Anomalously large amounts of hydrolytic enzymes are delivered to lipofuscin loaded lysosomes by Golgi apparatus to accelerate the degradation process although its efficacy and efficiency will not be improved due to the persistence of lipofuscin viability (Terman *et al.*, 1999; Cuervo and Dice, 2000). It was hypothesized that, QDs that were trafficked to these enlarged lysosomes containing higher amount of hydrolytic enzymes in senescent cells will be degraded at a faster rate than normal lysosomes in young cells. The ensuing rapid release of Cd$^{2+}$ and other byproducts from the QDs will increase the agitation on lysosomal lumen and destabilize it further by permeabilizing its membrane. Concurrently, accumulation of QDs at higher concentration in lysosomal compartment of senescent cells as shown in the colocalization experiment will cause larger quantity of Cd$^{2+}$ to be released into the lysosomal lumen and amplify the antagonistic effects (section 5.3.5).

Numerous studies have reported on the deleterious effect that lipofuscins and artificial lipofuscins had on the cell’s viability (Brunk and Terman, 2002; Hohn *et al.*, 2012). Elevated cellular oxidative stress was the measured response that links lipofuscin enriched cells to its reduced viability and the catalysis of Fenton reaction by metal containing lipofuscin was the ascribed mechanism. Research have also shown that lipofuscin was a source of oxidant in cytoplasm and its containment in autophagosomes and/or lysosomes had reduced its toxic effect (Hohn *et al.*, 2012). Hence, translocation of lipofuscin into cytosol in tandem with hydrolytic enzymes and Cd$^{2+}$ after lysosomal rupture will greatly enhance the overall toxicity and accelerate the cell death.
The data have also suggested that, LMP induced necrosis as the primary mode of senescent cell death was actuated by 30nM QD-PEG and 45nM QD-HC but not 30nM QD-HC. Based on the cytotoxicity graph, the increase in cell mortality occurred exponentially after a few hours of incubation with 30nM QD-PEG and 45nM QD-HC, which is a hallmark of LMP induced necrosis (Figures 6.15 & 6.16). However, similar exponential shift in cell death was not observed in 30nM QD-HC treated senescent cells; only gradually rising cell death curves as a function of time. Treatment with 30nM QD-PEG and 45nM QD-HC had reduced the total cell population to nil within 16 hours in both IMR90 and CCD841CoN senescent cells, while at the same duration 30nM QD-HC had only managed to inhibit 10-13% of the senescent cell population. At the end of incubation period of 24 hours, cell mortality was only 51-53% for 30nM QD-HC treated senescent cells of both types, demonstrating that the toxic effect exerted by the nanoparticle did not adhere to the same pattern as 30nM QD-PEG and 45nM QD-HC in terms of magnitude and prompt.

Moreover, in the lysosomal alkalization experiment, reduction in the toxicity levels for 30nM QD-HC treated senescent cells of both types were only 35-38%, compared to the controls, which was significantly lower than 30nM QD-PEG and 45nM QD-HC at percentages of 75-78% and 80-82% respectively for both the senescent cell types. Thus, suggesting that the induction of senescent cell death by 30nM QD-HC was not strictly via LMP induced necrosis.
Current attempt at explaining the modality of senescent cell death that was actuated by 30nM QD-HC begins with the investigation on the major constituent of the QD itself; the Cd$^{2+}$. To study the effect of Cd$^{2+}$, the senescent cells were incubated with cadmium chloride (CdCl$_2$) at a concentration that was slightly higher than the concentration of Cd$^{2+}$ contained within 30nM QD-PEG; the concentration at which the total cell population were inhibited at the shortest amount of time. The presumption was that, if the QDs were degraded in its entirety at this concentration, the maximum amount of Cd$^{2+}$ constituting the QDs will be released into either lysosomal lumen or cytosol and its effects on the cells can be observed. For comparative analysis of cytotoxicity, the senescent cells were treated with supernatants that were collected from 30nM QD-PEG, 30nM QD-HC and 45nM QD-HC after incubation in simulated lysosomal fluid for 24 hours to mimic the QD’s lysosomal digestion process. The results have shown that the highest toxic effect on the cells were induced by CdCl$_2$ (375 µM), followed by supernatants from 30nM QD-PEG, 45nM QD-HC and 30nM QD-HC, respectively (Figures 6.18 & 6.19).

To determine the type of senescent cell death that was actuated by CdCl$_2$ and the supernatants, the cultures were pre-treated with apoptosis inhibitor, ZVAD-FMK and autophagy inhibitor, 3-MA. The data have clearly indicated that autophagy was the primary mode of senescent cell death that was actuated by the calcium chloride and supernatant treatments. Moreover, the data also implies that the release of Cd$^{2+}$ from QDs increased in the order of 30nM QD-HC < 45nM QD-HC < 30nM QD-PEG, based on the toxic effects exerted by the supernatants. The order of Cd$^{2+}$ release from the QDs or its
quantity in the respective supernatants was anticipated based on the QD’s photostability analysis under different physiological conditions (section 3.3.5). The fact that the senescent cells were predisposed to autophagic cell death as a consequence of cadmium toxicity was anticipated due to two reasons: (1) several reports have shown that cadmium toxicity had induced autophagic cell death on different cell types (Meng et al., 2015; Yang et al., 2016), and (2) senescent cells expire naturally via autophagic cell death due to increased intracellular macroautophagic activity (Gosselin et al., 2009; Blagosklonny, 2013).

To delve deeper into the mechanism that links cadmium toxicity to autophagic cell death of senescent cells, the state of calcium regulatory system in senescent cells were reviewed. Basal cytosolic calcium levels were attenuated in senescent cells in response to the cellular transformation associated with ageing (Papazafiri and Kletsas, 2003); as such, sudden spikes in the intracellular calcium levels will be detrimental to these cells. Cadmium was shown to elevate intracellular calcium levels, hence disrupting the calcium homeostasis in senescent cells and consequently causing its death via autophagy (Wang et al., 2008). At the very least, two modes of action were implicated in cadmium induced autophagic cell death by way of calcium homeostasis disruption: (1) extracellular signal regulated kinase activation due to elevated calcium levels (Wang et al., 2008), and (2) reactive oxygen species generation due to elevated calcium levels (Shih et al., 2004).
Figure 6.18: (A) Temporal analysis and (B) quantification of cadmium and QD’s supernatant toxicity on normal and inhibitor pre-treated senescent IMR90 cells. ZVAD-FMK and 3-MA are inhibitors of apoptosis and autophagy, respectively. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with specific concentrations of cadmium chloride, QD-PEG and QD-HC nanoparticles. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Figure 6.19: (A) Temporal analysis and (B) quantification of cadmium and QD’s supernatant toxicity on normal and inhibitor pre-treated senescent CCD841CoN cells. ZVAD-FMK and 3-MA are inhibitors of apoptosis and autophagy, respectively. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation with specific concentrations of cadmium chloride, QD-PEG and QD-HC nanoparticles. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
Based on this experiment, it was also confirmed that the toxicity induced by 30nM QD-PEG and 45nM QD-HC did not result in autophagic cell death and LMP induced necrosis was indeed its primary mode of senescent cell death. This conclusion was based on the fact that, CdCl₂ concentration at similar amounts were only able reduce the cell population at approximately 32-38% in 24 hours; whilst, 30nM QD-PEG and 45nM QD-HC treatment were able to cause total death of cell population within 16 hours with the assumption that the QDs were totally degraded into Cd²⁺, which was not the case. More realistic comparison with the supernatants have confirmed even lower cell population reductions of 27% for IMR90 and 25.5% for CCD841CoN in 24 hours with 30nM QD-PEG’s supernatant treatment; whilst, it was 18% for IMR90 and 19.5% for CCD841CoN in 24 hours with 45nM QD-HC’s supernatant treatment. Similarly, toxicity that was induced by 30nM QD-HC on the senescent cells was much higher than CdCl₂ and the supernatants (Figures 6.18 & 6.19). However, the cell death pattern resembles more with autophagy as opposed to LMP induced necrosis (Figure 6.20).

The narratives explaining the toxicity on the senescent cells by QDs, CdCl₂, and supernatants should consider the cellular pathway(s) that were involved. In cultures that were treated with calcium chloride and supernatants, Cd²⁺ were trafficked into the cells via divalent metal transporter systems/channels that were integral to the mammalian cell system to absorb nutritional metals such as calcium and iron (Vesey, 2010).
Figure 6.20: Fluorescence micrographs of senescent CCD841CoN cells undergoing autophagic cell death due to nanoparticle exposure. Micrographs are ordered from top to bottom to correspond with the progression of incubation time. Experiments were carried out at 37°C for 24h. Cells were stained with CMFDA CellTracker fluorescent dye (green) and 30 nM QD-HC nanoparticles were marked in red. Magnification, 400X.
Disruption to the cellular processes or interference with cytoplasmic organelles by detrimental amounts of Cd\(^{2+}\) could give rise to toxicity that manifested as autophagic cell death (Wang et al., 2009). In cultures that were treated with QDs, the nanoparticles enter the cell via clathrin mediated endocytosis and were trafficked to lysosomes via endolysosomal pathway. After degradation or dissolution of QDs in the lysosomal lumen, the liberated Cd\(^{2+}\) as well as other lysosomal protein and enzymes will leak out into cytoplasm as a consequence of LMP. This mechanism is called lysosomal enhanced Trojan horse effect and is deemed more toxic to cells due to the release of other lysosomal substance into cytosol alongside Cd\(^{2+}\) could give rise to toxicity that manifested as LMP induced necrotic cell death (Ortega et al., 2014; Sabella et al., 2014).

In the case of 30nM QD-HC, toxicity induced by this nanoparticle had resulted in the demise of senescent cells via autophagy. As a consequence of lysosomal enhanced Trojan horse effect, Cd\(^{2+}\) from 30nM QD-HC were released into cell’s cytosol but the leakage from the lysosomes were much less pronounced because this nanoparticle had the lowest leakage of Cd\(^{2+}\) and the highest exocytosis rate compared to the other QDs as revealed by the current results. Abrupt leakage of Cd\(^{2+}\) will have the same effect on the senescent cells as did 30nM QD-PEG and 45nM QD-HC but the leakage from 30nM QD-HC was rather controlled due to less agitation and permeabilization of lysosomal membrane. However, as time progresses the breach in the lysosomal membrane will be larger and along with Cd\(^{2+}\), intact and partially degraded QD-HC will also escape into the cytosol and accelerate the death of senescent cells via autophagy. Metallomics study by Peng and co-researchers (Peng et al., 2015) have reported that QDs incubated with cells
had yielded different chemical derivatives of QDs as a consequence of lysosomal digestion. In the case of QD-HC, its partial degradation by lysosomes may infer that only the hard protein corona layer was digested and intact QD was the end product or semi intact QDs with exposed Cd\(^{2+}\) at the surface layer was the end product, both of which were equally toxic to the cells as Cd\(^{2+}\). Many studies have also reported on that toxic effects by intact QDs that had resulted in autophagic cell death (Winnik and Maysinger, 2013; Peynshaert et al., 2017). Hence, the increase in autophagic cell death in senescent cells that were treated with 30nM QD-HC relative to the CdCl\(_2\) and supernatants was due to the cumulative toxic effect that was actuated by Cd\(^{2+}\), intact and partially degraded QDs.

To gauge the response of young cells to cadmium toxicity, similar CdCl\(_2\) treatments were administered to replicating and contact inhibited cultures of both cell types. There was no significant difference in the toxic effect compared to the controls but there was a mean decrease of about 3-5\% in replicating and contact inhibited cells of both types (Figure 6.21). It was conjectured that the eliminated cells were of senescence origin because it coincides with the percentile range of senescent cells that were typically found within the young cell population of \textit{in vitro} cultures and also due to its increased susceptibility to cadmium toxicity. The data have shown that, at the same concentration of CdCl\(_2\), mortality in senescent cells were about 31-33\% but insignificant in young cells compared to the controls of both cell types. Cd\(^{2+}\) from the CdCl\(_2\) treated cells were trafficked via the divalent metal transporter systems/channels into the cell’s cytosol, hence directly disrupting the cellular processes at toxic concentrations. As such, detoxification of the heavy metal from cytosol will alleviate its cytotoxic effects and based on the results,
It was suggested that the young cells had an effective mechanism to counteract the Cd\textsuperscript{2+} interference, which may be lacking in the senescent phenotype.

It was hypothesized that metallothionein (MT) proteins played a vital role in the mitigation of cadmium associated toxic effects on the young cells. MTs are low molecular weight proteins that chelate Cd\textsuperscript{2+} from cytosol and translocate it to lysosomes after the autophagocytosis of Cd-MT conjugates (Klaassen and Liu, 1997). MT expression level in senescent cells were reported to be down-regulated with respect to the young cells and more pertinent to the current study was the fact that induction of MT after exposure to CdCl\textsubscript{2} was reduced in the senescent cells, thus making it more sensitive, to cadmium toxicity than the young cells (Malavolta \textit{et al.}, 2008; Mocchegiani \textit{et al.}, 2013). In a study performed on IMR90 cells, identical to the fibroblast model that was used in the current study, it was concluded that the induction of MT by CdCl\textsubscript{2} were reduced significantly and only a third of the MT proteins levels were detected in senescent cells compared to the young cells (Luce \textit{et al.}, 1993). MT proteins were also reported to affect the state of lysosomal health by enhancing its stability (Baird \textit{et al.}, 2006). Autophagocytosed MT proteins were able to reduce intralysosomal oxidative stress by binding to heavy metals that partake in fenton-type reaction, therefore reducing lysosomal membrane permeabilization.

Based on the CdCl\textsubscript{2} and QD toxicity results, it was presumed that even if Cd\textsuperscript{2+} were to escape from intact lysosomes of young cells after QD degradation, MT proteins
in these cells will effectively remove the ions from disrupting the cellular processes. On the other hand, the cumulative effect of reduced MT proteins and higher levels of lipofuscin in senescent cells will antagonize the lysosomal stability by increasing its oxidative stress. These deleterious effect was further aggravated by QD localization and degradation leading to increased leakage or rupture of the lysosomes. Moreover, the leaked Cd\textsuperscript{2+} into cell’s cytosol will not be effectively removed due to lower MT protein levels in the senescent cell.
**Figure 6.21**: Cell viability of young IMR90 and CCD841CoN cells treated with cadmium chloride. Toxicity measurement was performed using WST-1 colorimetric assay after 24h incubation. Data are represented as mean ± SD. n=6 for each group. Significant differences are marked as asterisks (*). * P < 0.05, ** P < 0.01 and *** P < 0.001. (n.s.) not significant.
6.4 Conclusion

Results from the present study have demonstrated that the QDs were not acutely toxic to young IMR90 and CCD841CoN cells. In contrast, the QDs were lethal to senescent cells of both types with varying degree at the same exposure time. Two major findings of the cytotoxic study were: (1) Susceptibility of senescent cells to the toxic effects of QDs were attributed to its phenotype and (2) Toxicity of QDs on the senescent cells were concentration dependent with higher concentration being more toxic to the cells and vice versa.

Based on the cytotoxic analysis of 30nM QD-PEG and 45nM QD-HC, the senescent cells perished by way of necrosis and upon further probing it was determined that lysosomal membrane permeabilization precipitated the cell death process. In the case of 30nM QD-HC, it was determined that the senescent cells expired gradually by way of autophagic cell death due to cadmium toxicity. In both cases, the common denominator was disruption to the lysosomal activity of senescent cells preceding the loss of its viability.

QD’s disintegration within the lysosomal compartment was determined to be the precursor event leading up to the binary cell deaths. The rate of QD’s disintegration was the determining factor for the mode of cell death and protein corona was found to effect this process in the current study. Deeper introspection had led to the discovery that protein corona had slowed down the QD’s disintegration and consequently had attenuated its cytotoxic potential.
CHAPTER SEVEN: CONCLUSION

7.1 General conclusion

In summary, the goal of the present study was to gain insight on the following subject matters: (1) to characterize and evaluate the impact of hard protein corona on the physicochemical properties of the QDs, (2) to establish \textit{in vitro} senescent cell models using fibroblast (IMR90) and epithelial (CCD841CoN) cells, (3) to determine the interaction and cytotoxic potential of pristine and hard corona coated QDs on young and senescent cells, and (4) to investigate the modality and mechanism of senescent cell death induced by the QDs.

To address the first point, the data have shown that hard protein corona had transformed the physicochemical properties of QDs and consequently its colloidal stability in a significant manner. At proportionate levels of nanoparticle concentration, hard protein corona had imbued distinct colloidal properties to the QDs that were better suited for nanomedical applications in terms of: (1) enhanced photostability at extreme pH conditions, (2) greater resistance to changes in extracellular medium that induces agglomeration and gravitational sedimentation, and (3) increased robustness to degradation and leaching of QDs’ core material at extreme pH conditions.

To address the second point, the results have shown that senescence induction of IMR90 and CCD841CoN cells were successfully accomplished by three distinguishing methods: (1) replicative senescence induction via serial passaging of cells, (2) oxidative
stress induction via hydrogen peroxide treatment, and (3) genotoxic stress induction via doxorubicin treatment. The difference among the aging methods were insignificant in terms of effectiveness, however the time interval for consummation was expectedly longer in replicative senescence due to propagation of cells.

To address the third point, the data have shown that QDs were not acutely toxic to young IMR90 and CCD841CoN cells. In contrast, the QDs were lethal to senescent cells of both types with varying degree at the same exposure time. QD-PEG were acutely toxic to senescent IMR90 and CCD841CoN cells, leading to lysosomal membrane permeabilization induced necrotic cell death within 24 hours of incubation. The senescent cells had divergent response to the toxic effects induced by QD-HC depending on its concentration. At similar concentration of QD-PEG, QD-HC had induced autophagic cell death due to cadmium toxicity and halved (49-51%) the senescent cell population. Only at much higher concentrations of QD-HC, lysosomal membrane permeabilization induced necrosis was observed, resulting in total death of senescent cell population. At all instances, the common denominator was the disruption to the lysosomal activity of senescent cells preceding the loss of its viability. QD disintegration within the lysosomal compartment was determined to be the precursor event leading up to the binary cell deaths. The rate of QD disintegration was the determining factor for the mode of cell death and protein corona was found to effect this process in the current study. Deeper introspection has led to the discovery that protein corona had delayed the QDs’ disintegration and consequently had attenuated its cytotoxic potential.
To address the fourth point, the data have shown that the transition of IMR90 and CCD841CoN cells into senescent state by way of replicative/premature senescence induction had resulted in weakened cells due to deterioration of organelles and disruption in cellular functions. The collective dysfunctionality of the senescence phenotype had increased its susceptibility to toxic effects by the QDs. Overall reduction in the endocytotic activity due to lowered ATP production by deteriorating mitochondria and more explicitly, attenuation in the rate of clathrin mediated endocytosis as a consequence of amphiphysin-1 protein down-regulation had increased the uptake of larger QD aggregates in the senescent cells. The internalization of larger QD aggregates coupled with senescent cell’s vast surface area had resulted in the accumulation of higher concentration of nanoparticles. At the intracellular level, QDs localization at higher concentrations in the enlarged and leaky lysosomes that were rich in lipofuscin pigments had resulted in further destabilization of this organelle; thus, its eventual collapse was accompanied by the release of its contents into the cell’s cytosol that concluded in LMP induced necrotic cell death. In the instance that abrupt lysosomal breakdown did not manifest, inadequate chelation or clearance of leaked Cd\(^{2+}\) due to attenuated production of MT proteins in the senescent cells had concluded in autophagic cell death. Therefore, the QDs had enhanced the deleterious effects on the functionally exhausted senescent cells until an irreversible phase had been reached whereby the damages done to the cells were irreparable and cell death was the only inevitable outcome.

With regards to the current state of knowledge, this aspect of nanotoxicology was not reported previously; hence the information derived from this research would be
valuable to the biomedical community. The findings from this study will benefit researchers in the field of nanomedicine to design their experiments more effectively after adjusting for protein corona influences and age related differences in studies featuring nanoparticle based drug delivery systems that were geared towards therapeutic or clinical applications. It might also explain some of the incongruences or inconsistencies in the results reported by previous studies. For instance, when conducting clinical trials on nanoparticle based drugs, the sample population comprising of different age groups might show highly varied effects to the same drug formulation administered. The present study had provided some clues on why the age of the test group might be a contributing factor. Therefore, steps can be taken to normalize the effects by reformulating dosages in accordance to the age groups, which will minimize the perturbation in the data obtained from clinical trials.

Another potential utility of the current finding lies in the mitigation of unwarranted cytotoxic effects on the cells due to therapeutic or clinical administration of nanoparticles. This can be achieved by pre-conditioning the nanoparticles in patients’ blood before intravenous administration to harvest protein corona adsorption from the blood plasma. However, this strategy might pose a problem for targeted drug therapy as the protein corona could modulate the physiochemical properties of the nanoparticles. On the other end of the spectrum, the selective toxicity exhibited by nanoparticles on the senescent cells can be exploited to target and annihilate the aging cells. Hence, these nanoparticles can be utilized as senolytic agents for anti-aging treatments provided that it discriminates between the cell types.
7.2 Suggestions for future studies

The scope of the current studies should be expanded to include different classes of nanoparticle in order to reach a better consensus on the effects of protein corona. By evaluating the effects of protein corona using a broad spectrum of nanoparticles, sweeping generalizations can be made about its effect rather than to delegate it as an anomalous occurrence. Similarly, different cell types should be included in the future studies to gauge the effects of protein corona in a more comprehensive manner.

In the current study, hard corona layer was comprised of proteins that were sourced from pooled human plasma; therefore, provisions should be made in the future studies to include human plasma that were derived from individual sources or groups of individuals based on the age groups. The basis for carrying out such a study is to emulate the blood plasma conditions of different age groups and its influence on the protein corona formation. For instance, higher level of protein molecules in human plasma of aged individuals exists in oxidized state that may affect the type, amount and affinity of protein adsorption towards the nanoparticles. Hence, protein corona profile of young and old blood donors might differ slightly and its impact on the cells should be analyzed comparatively. This is a step further from the present study in understanding the relationship between cell age and the effects of protein corona.
REFERENCES


Liu, X., Yang, W., Guan, Z., Yu, W., Fan, B., Xu, N. & Liao, D. J. 2018. There are only four basic modes of cell death, although there are many ad-hoc variants adapted to different situations. *Cell & bioscience*, 8(1), pp 6.


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